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EDITORIAL

REVIEWS

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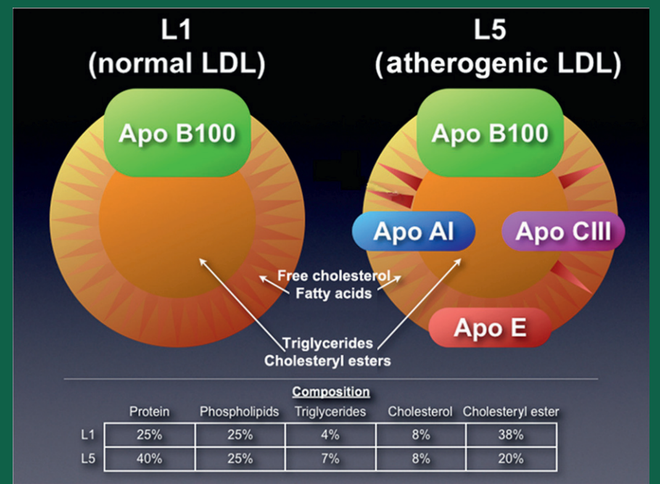

Composition

	Protein	Phospholipids	Triglycerides	Cholesterol	Cholesteryl ester
L1	25%	25%	4%	8%	38%
L5	40%	25%	7%	8%	20%

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Editorial

Cardiovascular disease and cancer progression—A brief insight

Apart from the environmental factors, the complex interaction of genes plays a central role in the development of diseases. Growing evidence indicates that death due to cardiovascular complications and cancer is increasing and survival rate decreasing due to the availability of fewer therapeutic options. The current issue of *Biomedicine* focuses on some of the risk factors that influence cardiovascular disease, and on the functional roles of androgenic signals, very long noncoding RNAs (vlncRNAs), and betel quid chewing in cancer progression.

The first paper reviews the current literature on angiotensin-converting enzyme inhibitory peptides, focusing on their structure–activity relationship and inhibitory mechanisms. In addition, an *in vitro* simulated gastrointestinal digestion model for assessing bioavailability and an *in vivo* examination of the antihypertensive effects of the peptides are also summarized.

The second review mainly focuses on the types of hyperlipidemia, digestion, and absorption of lipids, as well as their consequences on human health, and on potentially effective new therapeutic targets for treating hyperlipidemia.

Low-density lipoprotein (LDL) abnormality is a central cause of atherosclerosis and is associated with complications such as coronary artery disease. Negatively charged LDL (L5), a subclass of circulating LDL, is a nonoxidized or merely oxidized LDL, but it is potentially atherogenic. L5 was isolated from human plasma for mechanistic scrutiny. This review article elucidates the potential role of L5 in endothelial dysfunction and atherosclerosis formation.

Androgenic signals (androgen/androgen receptor; A/AR) function in a biphasic manner in the progression of hepatocellular carcinoma. In this review, the author discusses the roles of the A/AR in hepatic host immunity and the hepatic damage/regenerating state, and how the A/AR signals influence hepatitis B virus replication in cirrhotic livers.

Long noncoding RNAs (lncRNAs) and vlncRNAs, which are more than 5000 nt long, are associated with numerous biological functions. XIST, MALAT1, PCA3, PCGEM1, and PCNCR1 are some of the vlncRNAs expressed during the progression of cancer. This review focuses on vlncRNAs, a specific group of lncRNAs that are more than 5000 nt long, and explored their roles in the development of diseases.

Although extensive research findings have linked betel quid chewing with oral cancer and precancerous conditions, to date, no pharmacological or behavioral treatment exists for betel quid cessation. In this review, the author reports on the prevalence of betel quid use in Taiwan and the link between betel quid chewing, smoking, and oral cancer among Taiwanese men. The author also elaborately discusses the defaults about smoking and betel quid addiction.

Overall, this issue widens and deepens our knowledge of cardiovascular disease and cancer research by providing new perspectives. Further studies are required to facilitate the development of novel therapeutic applications to treat the abovementioned diseases.

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Review article

Angiotensin I-converting enzyme inhibitory peptides: Inhibition mode, bioavailability, and antihypertensive effects

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ABSTRACT

Bioactive peptides within the original food-derived proteins are inactive but can be activated by releasing them during food processing (by enzymatic hydrolysis or fermentation) or during gastrointestinal (GI) digestion. Among all the bioactive peptides, the antihypertensive peptides attract particular attention owing to the prevalence of high blood pressure, which plays an important role in cardiovascular diseases. These peptides have the ability to act as angiotensin I-converting enzyme (ACE) inhibitors. Previous studies have shown that the ACE inhibitory peptides functioned as competitive, noncompetitive, or uncompetitive inhibitors, and therefore, the structure–activity relationship of the peptides with various inhibition modes needs to be clarified. Besides, the ACE inhibitory activity of these peptides *in vitro* does not always suggest its antihypertensive effect *in vivo*, which is based on its fate to encounter GI enzymes and brush-border membrane peptidases, after oral administration. This paper reviews the current literature on ACE inhibitory peptides, focusing on the structure–activity relationship and inhibition mechanisms due to their inhibition modes. In addition, the *in vitro*–simulated GI digestion for assessing bioavailability and *in vivo* antihypertensive effects of the peptides are also summarized.

1. Introduction

Cardiovascular disease (CVD), a class of diseases that affects the heart and blood vessels, has been recognized as the biggest cause of death worldwide. High blood pressure, or hypertension, is a condition of sustained increase in blood pressure levels and is the primary risk factor for CVD. It is reported that >25% of the population worldwide (approximately 1 billion) had been affected by hypertension in 2000 and according to the findings of Kearney et al this figure is predicted to increase to 1.56 billion by 2025 [1].

The renin–angiotensin system is a hormone system that regulates blood pressure and fluid balance, and plays an important role in the pathophysiology of CVDs such as congestive heart failure and hypertension [2]. Plasma renin is responsible for the conversion of angiotensinogen released by

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the liver into angiotensin I, which subsequently undergoes proteolytic cleavage, in the presence of angiotensin I-converting enzyme (ACE), to form angiotensin II in the lungs. The hormone angiotensin II is a vasoconstrictor and its formation increases blood pressure. In addition, ACE also degrades bradykinin which has vasodilatation properties. Therefore, the use of ACE inhibitors is believed to lower hypertension and further prevent CVDs.

ACE inhibitors are originally synthesized from compounds found in pit viper venom, and synthetic ACE inhibitors such as captopril, enalapril, lisinopril, and ramipril are currently used in the treatment of hypertension in humans [3,4]. Although these synthetic inhibitors show a remarkable effect in treating hypertension, they also cause adverse side effects, such as cough, allergic reactions, taste disturbances, and skin rashes. Thus, the development of safe and natural ACE inhibitors is necessary for future treatment and prevention of hypertension.

Many studies have successfully produced and isolated ACE inhibitory peptides from various food proteins such as gelatin [5], milk [6], maize [7], sunflower [8], ovalbumin [9], and wheat germ [10]. These peptides have frequently been reported to act as competitive inhibitors of ACE [11–13]; however, in recent years, some noncompetitive and uncompetitive ACE inhibitory peptides have also been isolated [14–17]. Although some studies have demonstrated the relationship between ACE inhibitory activities and peptide structures, only few studies discussed about the activity and inhibition mode of these peptides. On the other hand, in order to reduce increased blood pressure levels after oral administration, the peptides possessing *in vitro* ACE inhibitory activity have to reach the target organ in the organism in an active form. However, because these peptides are degraded by gastrointestinal (GI) enzymes, there is an inconsistency between their *in vitro* ACE inhibitory activity and *in vivo* antihypertensive activity. Although some researchers have used *in vitro*-simulated GI digestion to evaluate the bioavailability and bioactivity of ACE inhibitory peptides [18–20], the correspondence between both *in vitro* and *in vivo* effects has not been investigated in many studies. In this review, the relationship between ACE inhibitory activity and inhibition mode is introduced first. Then, the discrepancy between *in vitro* and *in vivo* activity is examined in the view of bioavailability. Finally, some future perspectives of these peptides are also discussed.

2. Inhibition mode of ACE inhibitory peptides

Proteins are well-known precursors of a range of biologically active peptides. The biologically active (also called “bioactive”) peptides are derived from food proteins that have a physiological effect in the body in addition to their nutritional value. The fact that proteins are precursors of biologically active molecules is particularly attractive for the development of functional foods, because the bioactive peptides are commonly-used food ingredients and are of natural origin. As compared with chemosynthetic drugs, food protein-derived peptides can be used as potent pharmaceuticals as alternatives to synthetic drugs because of the increasing interest for safe and economical use of drugs. The bioactive peptides are activated when released from proteins by enzymatic or acidic hydrolysis, and their biological activity is determined by their native amino acid composition and sequence [21].

Since the isolation of the first ACE inhibitory peptide from snake venom [22], many other ACE inhibitory peptides have been discovered in the enzymatic hydrolysates of various food proteins, including animal-, plant-, and microorganism-derived peptides. Table 1 [12,16,17,19,23–27,30,31,33–36] shows a summary of ACE inhibitory peptides from different food proteins categorized by their inhibition mode.

2.1. *Competitive inhibitor*

The inhibition mode of ACE inhibitory peptides is evaluated using Lineweaver–Burk plots. The competitive inhibitors can bind to the active site to block it or to the inhibitor-binding site that is remote from the active site so as to alter the enzyme conformation such that the substrate no longer binds to the active site (Fig. 1) [23]. For example, NLP [24], NG [24], YN [25], LGFPTTKTYFPHF [26], VVYPWT [26], LNVPGEIVE [27], NIPPLTQTPV [27], DKIHPF [27], LF [28], WA [28], WM [28], were reported to be competitive inhibitors. A previous study has reported that the active sites of two domains of somatic ACE are structurally and functionally homologous to a dipeptidyl carboxypeptidase, and that the zinc-coordination geometry is critical for their hydrolytic action [3]. However, the two catalytic sites are differentially activated by chloride ions and the physiological substrate angiotensin I is preferentially bound to the C-domain catalytic site. The substrate also makes a contribution to the chloride-mediated activation of the active site. Therefore, these differences indicate that despite the higher level of primary sequence homology, structural and functional differences do exist between two active sites of C and N domains. Three subsites, S1 (antepenultimate), S1′ (penultimate), and S2′ (ultimate), with unshared distinct characteristics for the binding of C-terminal amino acids of substrates or inhibitor are located on two homologous active sites. For the inhibitor–enzyme binding and interaction, three main subsites on the active site of the enzyme with different amino acid sequence should be bound with the substrate. Binding of inhibitor or the natural substrate to the enzyme takes place predominantly via the C-terminal tripeptide residues. The peptides with high ACE inhibitory activity have Trp, Phe, Tyr, or Pro at their C terminus and the branched aliphatic amino acids at the N terminus, and ACE is known to have little affinity toward inhibitors with C-terminal dicarboxylic amino acid, such as Glu [29]. More specifically, the presence of aromatic amino acids, Pro, Ala, Val, and Leu are most favorable for the antepenultimate position (S1), while Ile is most favorable for the penultimate position (S1′). Pro and Leu in the substrate sequence are most favorable for the ultimate position (S2′) with regard to the affinity exerted on the enzyme [30,31]. However, there are several peptides such as NNTGHNFENTGEAM [32] and WM [28] that do not fit into the model.

2.2. *Noncompetitive inhibitor*

The noncompetitive inhibition system shows that both the inhibitor and the substrate can be bound to the enzyme at any given point of time. When both the substrate and the inhibitor

Table 1 – Protein-derived ACE inhibitory peptides categorized by inhibition modes.

Source	Preparation	Peptide	IC_{50} (μM)	Reference
1. *Competitive inhibitors*				
Soy	Alcalase	NLP	4.8	[23]
		NG	12.3	
Hard clam meat	Protamex	YN	51	[24]
Porcine hemoglobin	Pepsin	LGFPTTKTYFPHF	4.92	[25]
		VVYPWT	6.02	
Milk	Fermentation	LNVPGEIVE	300.1	[26]
		NIPPLTQTPV	173.3	
		DKIHPF	256.8	
Mushroom	Hot water extraction	VIEKYP	129.7	[30]
Chum salmon muscle	Thermolysin	LF	383.2	[27]
		WA	277.3	
		WM	98.6	
Marine rotifer	Alcalase	NNTGHNFENTGEAM	9.64	[31]
2. *Noncompetitive inhibitors*				
Tofuyo (fermented soybean)	Fermentation	IFL	44.8	[19]
		WL	29.9	
Hen ovotransferrin	Chymotrypsin	KVREGTTY	102.8	[33]
	ACE	KVREGT	9.1	
Oyster	Pepsin	VVYPWTQRF	66	[34]
Oyster	Trypsin	DLTDY	143	[35]
Algae protein waste	Pepsin	VECYGPNRPQF	29.6	[36]
Yellowfin sole frame	α-Chymotrypsin	MIFPGAGGPEL	26.4	[37]
Tuna frame	Pepsin	GNLGKTTTVSNWSPPKWKNTP	11.28	[12]
Chum salmon muscle	Thermolysin	FL	13.6	[27]
		AW	6.4	
3. *Uncompetitive inhibitor*				
Wakame	Protease S	IW	1.5	[16]
		FY	42.3	
		AW	18.8	
Human plasma	Trypsin	YLYEIARR	86	[17]

ACE = angiotensin I-converting enzyme; IC_{50} = median inhibitory concentration.

are bound, the enzyme–substrate–inhibitor complex cannot form a product but can only be converted back into the enzyme–substrate complex or the enzyme–inhibitor complex [33]. Some food protein–derived peptides were reported to be noncompetitive inhibitors of ACE. These include IFL [19], WL [19], KVREGTTY [34], KVREGT [34], VVYPWTQRF [20], DLTDY [35], VECYGPNRPQF [36], and MIFPGAGGPEL [37].

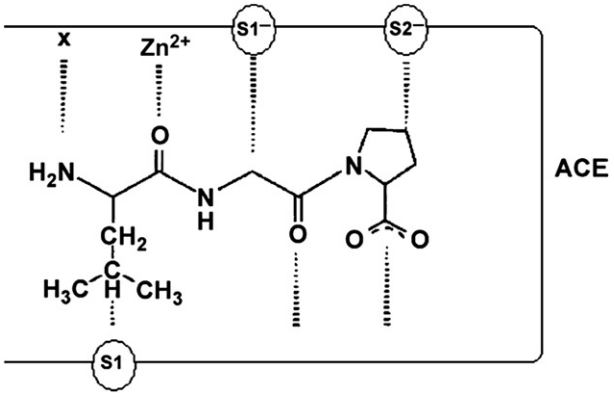

Fig. 1 – Active site of angiotensin I-converting enzyme (ACE) showing the interactions between ACE inhibitory peptides and ACE [23].

The inhibition site of these peptides is not specified because of the various structures of the peptides derived from different parent proteins, and the noncompetitive inhibition mechanism of ACE inhibitory peptides is not clear yet. In order to understand the inhibition mechanism, TPTQQS, a hexapeptide that acts as a noncompetitive inhibitor of ACE to prevent the formation of the reaction product His-Ala, was used to investigate the interactions between ACE, TPTQQS, and the nonphysiological substrate, hippuryl-His-Leu [38]. The results obtained showed that when ACE is in the unbound form, the zinc ion and HEXXH (the key amino acid residues of ACE active site) motif compose the complete active site of ACE, and hippuryl-histidyl-leucine can enter the active site and be converted into the reaction product. After TPTQQS enters ACE, the Thr1, Thr3, and Gln4 residues of TPTQQS allow the peptide to interact with the lid structure of testos ACE (tACE), and the C-terminal Ser6 pushes the zinc ion away from the active site through the coordination bonds between the Ser and the zinc ion, resulting in noncompetitive inhibition of ACE by TPTQQS (Fig. 2) [38]. Although the noncompetitive inhibition model of ACE using TPTQQS has been established, the other inhibitory peptides that function as noncompetitive inhibitors may not fit into this model because of their various peptide lengths and composition. Further investigations are being carried out to identify the relationship between the inhibition mechanism

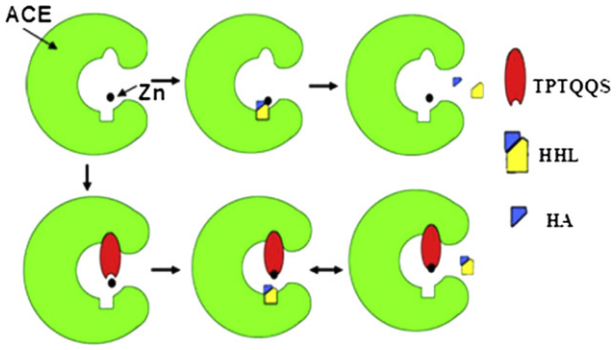

Fig. 2 – Model of the inhibition of angiotensin I-converting enzyme (ACE) by TPTQQS. The model shows that TPTQQS moves the zinc ion away from the active site to inhibit ACE [38]. HHL = hippuryl-histidyl-leucine.

and the structure of these peptides. In any case, this was the first study to report the noncompetitive inhibition mechanism of ACE inhibitory peptides, and this study has also provided fresh thoughts for designing drugs or functional foods against enzyme targets.

2.3. *Uncompetitive inhibitor*

In the uncompetitive inhibition system, the inhibitor can bind only to substrate–enzyme complex and decrease the maximum enzyme activity, so that it takes longer for the substrate or product to leave the active site. Peptides such as IW [16], FY [16], AW [16], and YLYEIARR [17] were reported to act as the uncompetitive inhibitors of ACE. However, the inhibition mechanism of this mode is not clear yet.

The fact that ACE is a dipeptidase makes it plausible for further hydrolysis of ACE inhibitory peptides and affects their antihypertensive activity *in vivo*. Depending on the outcome of GI digestion and other enzyme action *in vivo*, ACE inhibitory peptides can be classified as either true inhibitor (i.e., ACE inhibitory activity remains unchanged), prodrug (i.e., increased ACE inhibitory activity), or substrate (i.e., decreased ACE inhibitory activity) types of ACE inhibitors [39]. However, the active sites or these peptides which act as competitive, noncompetitive, or uncompetitive inhibitors are not specified, and the exact inhibition mechanism of ACE inhibitory peptides remains unclear. Further studies are necessary to figure out the correlation between the inhibition mode and the structure of the peptides.

3. Bioavailability

In order to administer the ACE inhibitory peptides orally in hypertensive patients, these peptides have to pass through the digestive tract to be absorbed through the intestinal epithelium. Digestion of proteins and peptides starts in the stomach by the action of pepsin at acidic pH, and then the polypeptides are further truncated by the pancreatic proteases, trypsin, α-chymotrypsin, elastase, and carboxypeptidases A and B at more alkaline pH. The *in vitro* bioavailability of the bioactive peptides is usually tested by sequential hydrolysis of pepsin and mimicking the actions of pancreatic enzymes so as to simulate the conditions of the GI. The inhibitory activity of some ACE inhibitory peptides was reported to decrease during simulated GI digestion. After sequential treatment with pepsin, chymotrypsin, and trypsin, the median inhibitory concentration (IC_{50}) values of IFL and WL (both isolated from tofuyo extract) against ACE varied from 44.8 to 117.9 μM and from 29.9 to 103.1 μM, respectively [19]. The ACE inhibitory activity of TQVY isolated from rice protein also showed a slight decrease after simulated GI digestion [40]. Some studies also demonstrated that several peptides are resistant to the digestive proteases, such as VIEKYP (from mushroom) [31], LNVPGEIVE, NIPPLTQTPV, DKIHPF (from fermented milk) [27], WVPSV, YTVF, VVYPW (from porcine hemoglobin) [41], and IPP, VPP (from β-casein) [42]. Furthermore, DLTDY was hydrolyzed by the simulated GI digestion to a shorter active form, DY, and the IC_{50} value against ACE decreased from 143 μM for DLTDY to 28 μM for DY [35]. In one of the studies, egg-derived ACE inhibitory peptides YAEERYPIL and RADHPFL were hydrolyzed to other active forms after simulated GI digestion [43]. There was also a study in which the peptide KVLPVPQ derived from β-casein showed low ACE inhibitory activity (IC_{50} = 1000 μM); however, the shorter peptide KVLPVP obtained after pancreatic digestion showed greater ACE inhibitory activity at the IC_{50} value of 5 μM [44]. In the same study, YKVPQL with strong ACE inhibitory activity (IC_{50} = 22 μM) failed to act as a potent ACE inhibitor with IC_{50} > 1000 μM after pancreatic digestion.

In general, Pro- and hydroxyproline-containing peptides are resistant to degradation by digestive proteases, such that the tripeptides with the C-terminal Pro–Pro are reported to be resistant to Pro-specific peptidases [45,46]. This might be the reason that some ACE inhibitory peptides derived from casein and gelatin have been shown to exert *in vivo* antihypertensive effect, as the Pro content in these two proteins is high.

4. Antihypertensive effect

The inhibitory activity of the peptides against ACE does not always correlate with their *in vivo* antihypertensive effects. There are two ways in which the ACE inhibitory peptides exert an antihypertensive effect *in vivo* after oral administration. First, the peptides retain their intact structure and second, they are hydrolyzed into active products, after the action of digestive enzymes, absorbed in the intestine and finally reach their target sites. For this purpose, *in vitro*–simulated GI digestion is a simple and cheap experimental method that is usually used for mimicking the *in vivo* effect. However, the relationship between the *in vitro* ACE inhibitory activity of the peptides via simulated GI digestion and *in vivo* antihypertensive activity is not clear.

An ACE-inhibitory peptide KVLPVPQ derived from casein was degraded to KVLPVP after the simulated GI digestion, and they showed the similar ACE inhibitory activity. After oral administration at a dose of 2 mg/kg, the two peptides KVLPVP and KVLPVPQ got a systolic blood pressure (SBP) reduction of 32.2 and 31.5 mmHg, respectively [44]. In the same study, YLVPQL possessing a strong ACE inhibitory activity was hydrolyzed by simulated GI digestion to YLVP with low ACE

inhibitory activity, and the YLVP showed no significant antihypertensive effect for SHR. Another ACE inhibitory peptide DLTDY with an IC_{50} value of 143 μM was degraded by simulated GI digestion to a dipeptide DL with an IC_{50} value of 28 μM. After both of these ACE inhibitory peptides were orally administrated to SHRs (at dosages of 8 and 10 mg/kg, respectively), their SBPs significantly decreased by about 15 and 20 mmHg at 3–6 and 3 hours [35], respectively. In another study, the ACE inhibitory activity of the peptide TQVY that had an initial IC_{50} value of 18.2 μM slightly decreased after simulated GI digestion. The peptide also showed the antihypertensive activity (by decreasing SBP to 40 mmHg) in SHR when a dosage of 30 mg/kg was administered 6 hours after the oral administration [40].

However, some studies showed inconsistent results. In one of the studies, two peptides YPI and RADHP obtained from the ACE inhibitory peptides YAEERYPIL and RADHPFL, respectively, by simulated GI digestion were not as active as ACE inhibitors ($IC_{50} > 1000$ and $= 153$ μg/mL), but showed a great antihypertensive effect on SHR at a dosage of 2 mg/kg 2–4 hours after oral administration (SBP: 31.6 and 34.0 mmHg, respectively) [43]. In another study, three ACE inhibitory peptides WVPSV, YTVF, and VVYPW had initial IC_{50} values of 0.368, 0.226, and 0.254 mg/mL, respectively [41]. After the simulated GI digestion, the ACE inhibitory activity of WVPSV and YTVF slightly increased, while that of VVYPW remained almost unchanged. Three hours after the oral administration of the former peptides, the SBPs of SHRs decreased by 22.5 and 18.5 mmHg, respectively, whereas after administration of the latter peptide it decreased only by 9.6 mmHg. The possible explanation is that these peptides need further activation by intestinal brush-border or plasma peptidases [43].

In fact, it is difficult to establish a model that directly correlates with *in vitro* ACE inhibitory activity and *in vivo* antihypertensive activity of peptides. The first reason is the bioavailability after oral administration, and the other is that some other antihypertensive mechanisms other than ACE inhibition may be of interest. Although the ACE inhibitory activity of the peptides through *in vitro*–simulated GI digestion may not always ensure their *in vivo* antihypertensive activity, simulated GI digestion is still a good method for preliminary test that is carried out to understanding the possible changes of the peptide structure and screening the potent antihypertensive peptides. Some studies have demonstrated the existence of vasorelaxant peptides that exert their effects through the stimulation of opioid receptors [47]. Moreover, these peptides exhibited a direct or indirect action on vascular smooth muscles [48,49]. In addition, strong evidence indicates that oxidative stress and associated oxidative damage are mediators in cardiovascular pathologies, and thus, antioxidant activity can also be responsible for antihypertensive effects [50].

5. Conclusions and future perspectives

Much work has been done with food protein–derived ACE inhibitory peptides and evidence of their *in vivo* antihypertensive effect has been built in animal and clinical studies. However, the ACE inhibition mechanisms of the peptides with different inhibition modes are still vague due to the various lengths and sequences of these peptides derived from different parent proteins. It is difficult to establish a model and a rule to describe the structure–activity relationship. "BIOPEP" database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) is responsible for collecting all the information about bioactive peptides from academic literature, and currently the database lists the sequences of 556 ACE inhibitory peptides. Further studies on the establishment of the model of structure–activity relationship are necessary, which can be achieved by using computing methods.

The most challenging task in the antihypertensive peptide researches is the establishment of a detection model for the identification of possible mechanisms by which they can exert *in vivo* antihypertensive activity. Although the simulated GI digestion is a kind of model mimicking the actions of human GI enzymes, the antihypertensive effect of the peptides released from the ACE inhibitory peptides through simulated GI digestion is not guaranteed to be similar to that observed in the GI tract *in vivo*. Therefore, the possible strategies for increasing the resistance to digestive enzymes and cellular permeability of antihypertensive peptides should be also investigated.

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Review article

Therapeutic approaches to drug targets in hyperlipidemia

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ABSTRACT

Hyperlipidemia is a metabolic syndrome characterized by diverse lipid profiles (e.g. hypercholesterolemia, hypertriglyceridemia, and familial combined hyperlipidemia) and may have significant adverse effects on health (e.g. atherosclerosis, cardiovascular diseases, diabetes, insulin resistance, obesity). Both genetic and environmental components are associated with hyperlipidemia sub-types. Effective drugs targeting hyperlipidemia sub-types are thus required. In the present review, we mainly focus on types of hyperlipidemia, digestion, and absorption of lipids as well as on their consequences on human health and on potential effective drug targets against hyperlipidemia. Omega-3 fatty acids have favorable effect on reducing postprandial triglyceride levels and will be beneficial if combined with statins.

1. Introduction

Hyperlipidemia is a heterogeneous disorder commonly characterized by an increased flux of free fatty acids (FFAs), raised triglycerides (TGs), low-density lipoprotein-cholesterol (LDL-c) (aka "bad cholesterol") and apolipoprotein B (apoB) levels, as well as by a reduced plasma high-density lipoprotein (HDL)-cholesterol concentration (aka "good cholesterol"), because of metabolic effects, or dietary and lifestyle habits [1]. The lipid abnormality in hyperlipidemia is an increase in circulating (nonesterified) FFAs originating from adipose tissue, and an inadequate esterification and FFA metabolism [2]. The reduced retention of fatty acids (FAs) by adipose tissue leads to an increased flux of FFA returning to the liver, which stimulates hepatic TG synthesis, promoting the production of apoB and the assembly and secretion of very low-density lipoprotein (VLDL). When plasma TG concentration subsequently increased, TG-rich HDL particles are formed and undergo catabolism. Elevated VLDL particles are lysed and hence fail to bind efficiently to LDL receptors, while the exchange of cholesterol esters with TGs forms TG-rich lipoproteins, resulting in formation of small dense LDL-c particles [3,4]. A strong association exists between elevated LDL-c levels and increased incidence of coronary artery disease [5]. The development of atherosclerotic plaques is associated with elevated levels of LDL-c, reduced receptor-mediated clearance, increased arterial wall retention and an increased susceptibility [6]. Cardiovascular risk factors such as hyperlipidemia,

Abbreviations: ACAT, Acyl-Co A: cholesterol acyltransferase; AMPK, AMP-activated protein kinase; apoB, Apolipoprotein B; ATP III, adult Treatment Panel III; CETP, cholesteryl ester transfer protein; CM, chylomicrons; DGAT, diacylglycerol acyltransferase; FCH, familial combined hyperlipidemia; FFA, free fatty acid; HC, hypercholesterolemia; HDL, high-density lipoprotein; HTG, hypertriglyceridemia; LDL, low density lipoprotein; NCEP, National Cholesterol Education Program; PPAR, peroxisome proliferator-activated receptors; TG, triglyceride; VLDL, very low density lipoprotein.

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hypertension, and thrombosis contribute to the underlying mechanisms of atherosclerotic disease, promoting endothelial dysfunction, oxidative stress, and proinflammatory pathways to peroxidation [4,6]. Lipid guidelines from the National Heart Foundation of Australia place great emphasis on LDL-c and HDL-c as atherogenic and antiatherogenic components, respectively. Indeed, high LDL-cholesterolemia is considered as one of the major modifiable risk factors for coronary heart disease, which continues to be the leading cause of death and morbidity in the United States [7]. Conversely to the Australian lipid guidelines, the Adult Treatment Panel III (ATP III) guidelines of the US National Cholesterol Education Program (NCEP) place greater emphasis on TG levels [4,8]. According to the National Health and Nutrition Examination Survey III, 24% of individuals aged >20 years had metabolic syndrome [9]. Metabolic syndrome is characterized by the coexistence of hyperinsulinemia, obesity, dyslipidemia, and hypertension. Dyslipidemia, the hallmark of the metabolic syndrome, is summarized by: (1) increased flux of FFAs; (2) raised TG values; (3) low HDL-c values; (4) increased of LDL-c values; and (5) raised apoB values [10]. Dyslipidemia is an independent risk factor for cardiovascular disease [11]. Low HDL-c and hypertriglyceridemia (HTG) have been found to be independently and significantly related to myocardial infarction/stroke in patients with metabolic syndrome [12]. The combination of high fasting glucose and low HDL-c were shown to have primary predictive ability for coronary heart disease [13]. Dyslipidemia may be caused by a combination of overproduction of VLDL, apoB-100, decreased catabolism of apoB containing particles, and increased catabolism of HDL-apoA-I particles. Insulin resistance may be the consequence of this abnormality [1]. Dyslipidemia may arise from genetic components (e.g. mutated LDL receptors, mutated apoB-100, mutated proprotein convertase subtilisin/kexintype-9) [14], with or without environmental component (e.g. improper diet, familial history of hypercholesterolemia, hyperlipidemia, and/or hypertriglyceridemia) [15]. Causes of secondary hyperlipidemia include diabetes, hypothyroidism, obstructive liver disease, chronic renal failure, and drugs that increase LDL cholesterol and decrease HDL cholesterol, such as progestine and corticosteroids [16].

2. Digestion and absorption of lipids

Lipid digestion begins in the oral cavity by the use of lingual lipase, an enzyme secreted by lingual gland in the tongue, and continues in the stomach with the both lingual and gastric enzymes. Lipids undergo emulsification in the stomach under the influence of peristalsis. Fine lipid droplets enter the duodenum, where they mix with bile and pancreatic juice to undergo marked changes in physical and chemical form. For absorption across the intestinal walls, hydrolysis and micellization take place in duodenum [17,18]. Diacylglycerol and FFAs are the major digestion products of this gastric phase, and facilitate the intestinal phase of digestion acting as emulsifying agents [19]. Pancreatic lipase cleaves the TG, yielding 2-monoglycerides (2-MGs) and FFAs. Pancreatic cholesterol esters hydrolase completely hydrolyzes cholesterol esters into FFAs and free cholesterol [20]. Dietary phospholipids are hydrolyzed by activated pancreatic phospholipase A2, yielding 1-lysophospholipids and FFAs [21]. FFAs and 2-MGs enter into bile micelles, which helps polar lipids to go through the unstirred water layer and reach the microvillous membrane where they are absorbed. Absorbed lipids are re-esterified to newly form TGs and in the smooth endoplasmic reticulum (ER). TGs can be synthesized via 2-MG or via 3-glycerol-phosphate. TGs, phospholipids, cholesterol, and apoproteins are used to synthesize chylomicrons (CMs), which are secreted to the lymph, and then to the blood stream through the thoracic duct. In the peripheral tissues, they are cleaved by lipoprotein lipase losing TG and giving CM remnants, which are taken up by the liver [21–23].

3. Hyperlipidemia profiles/sub-types

The classification of hyperlipidemia according to WHO is in Table 1 [17] and the constitution, composition and role of lipids in Table 2 [23].

3.1. *HTG*

Plasma TGs represent an important mechanism of whole body fatty acid delivery for tissue utilization or storage [23,24]. HTG is defined as an abnormally high concentration of TG in the blood. According to the NCEP ATP III guidelines, a normal TG level is <150 mg/dL [17]. In the United States, the prevalence of HTG, defined as a TG level >150 mg/dL, is 30%.

HTG is a risk factor for pancreatitis and it accounts for 1% to 4% of cases of acute pancreatitis [25]. HTG may be primary or secondary in nature. Primary HTG is the result of various genetic defects while the secondary causes are high fat diet, obesity, diabetes, hypothyroidism, and certain medications [26].

4. Patterns of HTG

Familial HTG is commonly seen in clinical practice and can have various lipid patterns [27].

4.1. *Hyperlipoproteinemia*

Most commonly, patients demonstrate type IV hyperlipoproteinemia, which includes elevated TG levels (250–500 mg/dL) and elevated VLDL levels that transport them, whereas normal LDL-c and apoB levels are observed [28].

4.2. *Chylomicronemia syndrome*

Familial chylomicronemia syndrome is a rare disorder of lipoprotein metabolism due to familial lipoprotein lipase (LPL) or apolipoprotein C-II deficiency or the presence of inhibitors to lipoprotein lipase [29]. The chylomicronemia syndrome is a disorder characterized by severe HTG and massive accumulation of CMs in plasma [30]. Finally, HTG may contribute to additional pathologic processes associated with metabolic syndrome and cardiovascular risk, including increased

Table 1 – World Health Organization (modified Fredrickson) classification of hyperlipidemias [17].

Type	Total cholesterol	LDL cholesterol	Plasma TGs	Lipoprotein abnormality	Primary causes	Secondary causes
I	Elevated	Low or normal	Elevated	Excess chylomicrons	Lipoprotein lipase deficiency, apoC-II deficiency	Systemic lupus erythematosus
II a	Elevated or normal	Elevated	Normal	Excess LDL	Familial hypercholesterolemia	Hypothyroidism
II b	Elevated	Elevated	Elevated	Excess LDL and VLDL	Familial combined hyperlipidemia	Nephrotic syndrome, diabetes, anorexia nervosa
III	Elevated	Low or normal	Elevated	Excess chylomicron remnants and Intermediate density lipoproteins	Familial type III Hyperlipoproteinemia	Hypothyroidis diabetes, obesity
IV	Elevated or normal	Normal	Elevated	Excess VLDL	Familial combined hyperlipidemia, Familial Hypertriglyceridemia	Diabetes, chronic renal diseases
V	Elevated	Normal	Elevated	Excess chylomicrons and VLDL	Familial hypertriglyceridemia, apoC-II deficiency	Alcohol, diuretics, β blockers, oral

apoC-II = apolipoprotein-C II; LDL = low-density lipoprotein; TG = triglyceride; VLDL = very low-density lipoprotein.

coagulability, impaired fibrinolysis, impaired endothelial function, and increased inflammation, although this remains uncertain [27,28,31].

4.3. HC

HC is one of the major causes of atherosclerosis and characterized by elevation of total cholesterol and usually normal levels of TG [32,33]. The population is considered to be unhealthy when its plasma concentration exceeds 5 mM and the incidence of CHD is usually low where plasma cholesterol concentration is low [34]. HC usually results from nutritional factors such as obesity and diet high in saturated fats along with genetic causes. The deficiency of adaptor protein Dab 2, or the clathrin coat adaptor AP-2 also leads to HC [32,35]. Patients with HC have plasma TG concentration of >10 mM owing to increase in both CMs and VLDL and, in such patients, plasma shows milky appearance [32]. Familial HC comprises a group of genetic disorders characterized by elevated plasma concentrations of LDL-c and premature cardiovascular disease due to a defective (mainly hepatic) metabolism of LDL [36]. Major genetic backgrounds of familial HC include loss-of-function mutations in the genes of LDL receptor, its ligand apoB or, gain-of-function mutations in the facilitator gene for hepatic LDL recepor degradation, the proprotein convertase subtilisin/kexin type-9 [35,37]. LDL receptors are predominantly found on hepatocytes and steroid hormone producing cells and are responsible for removal of cholesterol carrying LDL from plasma by a process of receptor-mediated endocytosis. The most important feature of untreated familial HC is the development of premature and extensive atherosclerosis leading to coronary artery diseases [38].

4.4. *Familial combined hyperlipidemia*

Familial combined hyperlipidemia (FCH) is the most common genetic hyperlipidemia in man and affects up to 5% of the general population [39]. HC, HTG, and elevated levels of apoB are the characteristics of FCH [40]. Other phenotypes of FCH are elevated levels of both LDL-c and VLDL, the presence of small dense LDL, and decreased levels of HDL-c. In addition, FCH is associated with obesity and insulin resistance [41,42]. Obesity results in an increase in number and size of adipocytes, which secrete leptin, a hormone involved in the regulation of the energy expenditure and appetite via hypothalamic receptors [43,44]. Both obesity and insulin

Table 2 – The constitution, composition, and role of lipids [23].

Constituent	Composition	Effect/role
Lipoproteins Chylomicrons	95% TG and 5% cholesterol	Mobilize dietary lipids, deliver dietary triglycerides to adipose tissues, muscles and dietary cholesterol to liver
VLDL	80% TG and 20% cholesterol	Transport triglycerols to extra hepatic tissues
IDL	50% TG and 50% cholesterol	They are either converted to LDL or taken up by the liver
LDL	10% TG and 90% cholesterol	Principal plasma carriers of cholesterol for delivering to peripheral tissues
HDL	5% TG and 95% cholesterol	The apolipoprotein-E in HDLs leads to an increase uptake of cholesterol and its catabolism by the liver to lower the levels of intracellular cholesterol

HDL = high-density lipoprotein; IDL = intermediate-density lipoprotein; LDL = low-density lipoprotein; TG = triglyceride; VLDL = very low-density lipoprotein.

resistance are characteristics of FCH, and therefore, it is likely that leptin is elevated in people with FCH [45]. Recently, in a large observational study, the calculated plasma non-HDL-c concentration was a stronger predictor of cardiovascular events than plasma cholesterol alone [46,47]. Partially lipolyzed TRL remnants (i.e. remnant-like particle cholesterol) are considered to be more atherogenic than larger newly secreted TRL because they can more readily penetrate the endothelial lining of the arterial wall [48]. In the metabolic syndrome, elevated levels of remnant-like particle cholesterol were a risk factor for cardiovascular disease and endothelial dysfunction, a predictor of coronary events [39,49]. FCH might be the result of a combination of an increased production of VLDL particles together with disturbances in their lipoprotein catabolism, such as a decreased LPL-activity [41]. The resulting partially hydrolyzed TG-rich remnant particles are more atherogenic than larger (newly-secreted) TG-rich lipoprotein particles, since the particles are smaller and thereby able to penetrate the endothelial barrier more easily [48,50]. A striking feature of FCHL is the presence of small and dense LDL particles, possibly consequent to hepatic overproduction of apoB [51–53]. Nondenaturing polyacrylamide gradient gel electrophoresis, which separates lipoprotein particles according to their size, has shown that the majority of the population can be characterized into two distinct, genetically determined, LDL subclass phenotypes [48]. Phenotype A is the most common phenotype and is found in individuals with a predominance of large LDL particles, whereas those with a predominance of small LDL particles have phenotype B [54]. Phenotype B often coexists with other lipoprotein abnormalities, notably raised plasma TGs and low HDL-c, in a condition that has been called 'atherogenic lipoprotein phenotype' [55]. Several regions on chromosomes including 2p, 6q, 8p, 9p, 10p, 11p, 16q, 19q, and 21q, have been reported to be associated with FCH [56]. The association of upstream stimulatory factor 1 with FCH however, was strongest in males with increased levels of TGs [57].

5. Current drug targets against hyperlipidemia

Conventional therapy for hyperlipidemia is as listed in Table 3 [28,58–62].

5.1. *Activators of peroxisome proliferator-activated receptor*

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily that function as fatty acid-activated transcription factors [63]. PPARs are regulators of numerous metabolic pathways; hence there is huge increase in the development and use of agonists of these receptors as therapeutics for diabetes, dyslipidemia, and atherosclerosis [64]. Three different PPAR genes (α, β/δ, and γ) have been identified, each isotype displaying distinct patterns of tissues distribution and specific pharmacological activators, performing their distinct functions in different cell types [65]. PPARα is mostly expressed in the tissues involved in lipid oxidation, such as liver, kidney, skeletal, cardiac muscle, and adrenal glands. PPARα potentiates FAs oxidation in the liver, heart, kidney, and skeletal muscle. Activation of PPARα leads to an increase in expression of lipoprotein lipase and apoA-V and to a decrease in hepatic apoC-III. These actions lower plasma TGs in chylomicrons and VLDL particles, thus liberating FAs, which are taken up and stored as fat in adipocytes or metabolized in skeletal muscle [66]. In addition, PPARα activation increases hepatic apoA-I and -II expression, which raises HDL cholesterol levels, and promotes HDL-mediated cholesterol efflux from macrophages by inducing ATP-binding cassette A1 transporter [67]. PPARγ is expressed in adipose tissue, macrophages, and vascular smooth muscles, while PPARδ is mainly expressed in skeletal muscle and adipose tissues [68]. PPARβ/δ is best known for its role in skin homeostasis, and has recently been shown to play a role in HDL metabolism [64]. A combination of PPARα and PPARγ agonists would be expected to achieve beneficial effects on restoring metabolic disorders. Hence, a number of PPARα/γ dual agonists have been designed and developed. However, recently identified PPARα/γ dual agonists were ineffective because of undesirable side effects during preclinical or clinical trials. For example, muraglitazar, a synthetic PPARα/γ dual agonist, was aborted during clinical trials because of increased mortality, fluid retention, edema, and cancer [69]. PPARα regulates genes involved in FA uptake, β-oxidation, and ω-oxidation and down-regulates apolipoprotein C-III, a protein that inhibits TG hydrolysis by lipoprotein lipase, and it also regulates genes involved in reverse cholesterol transport, such as apolipoprotein A-I and A-II [68]. PPARα and PPARγ are the molecular targets of number of marketed drugs such as fibrates, the activator of PPAR α and the thiazolidinediones, the activators of PPAR γ [59].

5.2. *Cholesteryl ester transfer protein inhibitors*

Cholesteryl ester transfer protein (CETP) is a plasma glycoprotein that facilitates the movement of cholesteryl esters and triglycerides between the various lipoproteins in the blood by mediating the transfer of cholesteryl esters from the cardioprotective HDL-c to the proatherogenic LDL-c and VLDL-c [70]. Thus, the movement of cholesteryl esters from HDL-c to LDL-c by CETP has the overall undesirable effect of lowering HDL-c. It therefore follows that inhibition of CETP should lead to elevation of plasma HDL-c and lowering of plasma LDL-c, thereby providing a therapeutically beneficial plasma lipid profile [71]. Elevation in HDL levels is equally favored by diminished CETP-mediated transfer of CE and HDL to atherogenic acceptor lipoproteins (i.e. VLDL, LDL). Elevated CETP activity is a major player whose action underlies the atherogenic particle profile of both LDL and HDL in Type II diabetes [72]. Inhibition of CETP, a key protein involved in reverse cholesterol transport, can consequently lead to increases in HDL-c levels and thus, is under evaluation as an anti-atherogenic strategy. To date, anacetrapib demonstrates the greatest HDL-c raising and LDL-c lowering potential [73]. There are three CETP inhibitors that have been used in clinical trials. Torcetrapib was the first to go into human trials but was discontinued in Phase III because of excessive rates of mortality in the ILLUMINATE (investigation of lipid level

Table 3 – Pharmacotherapy of hyperlipidemia [28,58–62].

Drugs	Mechanism of action	Use	Effect on lipoproteins	Adverse effects
Statins Lovastatin (20–80 mg) Pravastatin (20–40 mg) Simvastatin (20–80 mg) Atorvastatin (10–80 mg) Fluvastatin (20–80 mg)	By inhibiting conversion of 3-hydroxy-3-methylglutaryl-coenzyme A-CoA to mevalonate.	Type IIa	LDL decreases 18–55% HDL increases 5–15% TG decreases 7–30%	SGOT, SGPT, Myositis, Lens opacity, Myopathy, Headache GI complaints, Increase liver enzymes Rhabdomyolysis Impaired cognitive function
Bile acid sequestrants Cholestyramine (4–16 g) Colestipol (5–20 g) Colesevelam (2.6–3.8 g)	By interrupting enterohepatic recycling of bile acids. FXR mediated CYP7A repression	Type IIa	LDL decreases 15–30% HDL increases 3–5% TG no change or increases	Constipation and bloating, Hemorrhoidal bleeding Dry flaking skin Gallstone Myopathy Flatulence
Fibric acid derivatives Gemfibrosil (600 mg) Fenofibrate (200 mg) Clofibrate (1000 mg)	Increase lipolysis of triglycerides via lipoprotein lipase. Act as agonist for PPAR-α, resulting in increased expression of lipoprotein lipase and inhibition of apolipoprotein-C-III gene transcription	Types III and IV	LDL decreases 5–20% HDL increases 10–20% TG decreases 20–50%	SGOT, SGPT, Myositis Gallstone Arrythmias
Nicotinic acid Immediate release (1.5–3 g) Extended release (1–2 g) Sustained release (1–2 g)	By decreasing flux of FFA to the liver. Through Gi coupled receptor (GPR109A, PUMA-G, HM74) By noncompetitive blocking of DGAT2	Types IIa and IV	LDL decreases 5–25% HDL increases 15–35% TG decreases 20–50%	Flushing SGOT, SGPT Tachycardia Pruritus Glucose intolerance Hyperuricemia Nausea Diarrhea Hepatotoxicity

DGAT = diacylglycerol acyltransferase; FFA = free fatty acid; HDL = high-density lipoprotein; LDL = low-density lipoprotein; PPAR = peroxisome proliferator-activated receptor; SGOT = serum glutamic oxaloacetic transaminase; SGPT = serum glutamic pyruvate transaminase; TG = triglyceride; VLDL = very low-density lipoprotein.

management to understand its impact in atherosclerotic events) trial. Anacetrapib, which has a similar structure to torcetrapib but does not share its properties when it comes to the effects on aldosterone production, is presently in Phase III research. Dalcetrapib, which is structurally different than torcetrapib, is currently undergoing cardiovascular outcomes trials [74]. 2-Arylbenzoxazole, [75], tetrahydrochinoline (BAY 38-1335) [76], chromanol derivatives, and 2-(4-carbomylphenyl) benzoxazole are under development as CEPT inhibitors [77,78] .

5.3. *Cholesterol absorption inhibitors*

Ezetamibe is the only drug currently available from this class whose mechanism of action involves inhibition of dietary cholesterol absorption without affecting the absorption of fat-soluble vitamins, triglycerides, and bile acids [59,62,79]. Ezetamibe binds to cholesterol transporter NPL1L1 (Niemann-pick C1-like1) protein in the brush border of intestine as well as in hepatocytes [59,80]. Decrease in cholesterol absorption leads to compensatory up-regulation of LDL receptors on the cell surface and increased LDL cholesterol uptake into cells and decreases blood LDL cholesterol content [59,62]. Ezetamibe also exerts anti-inflammatory effect and also appears to improve renal function [81]. Some side effects of ezetamibe are diarrhea, abdominal pain, arthralgia, backache, myalgia, headache, sinusitis, hepatitis, aphylaxis, myopathy, and rhabdomyolysis [62]. This drug is contraindicated in active liver diseases [62]. Ezetimibe is primarily metabolized in the small intestine and liver via glucuronide conjugation with subsequent biliary and renal excretion [82]. After oral administration, ezetimibe is absorbed and extensively conjugated to a pharmacologically active phenolic glucuronide (ezetimibe-glucuronide), the drug and its metabolite have a half-life of approximately 22 hours [83].

5.4. *Cholesterol O-acyltransferase inhibitors*

Acyl-CoA: cholesterol O-acyltransferase (ACAT) is an important enzyme involved in re-esterification of absorbed cholesterol within enterocytes [84]. It is involved in the cholesterol metabolism in macrophages, liver, intestine and adrenal cortex and is believed to be involved in secretion of VLDL from liver and development of atherosclerotic lesion [85]. Two ACAT enzymes have been identified, ACAT1 and ACAT2. ACAT1 is found in the ER throughout the body, while ACAT2 is found in the ER of liver and intestinal tissues and may be responsible for the formation of cholesteryl esters [85]. In theory, inhibition of ACAT1 could prevent the transformation of macrophages into foam cells in the vessel wall and, thereby, slow the progression of atherosclerosis and prevent the development of vulnerable plaque and inhibition of ACAT-2 could decrease serum lipid levels by reducing the synthesis of lipoproteins [70]. HL-004 has been found preclinically to be an effective ACAT inhibitor [86]. Presently, specific ACAT 2 inhibitor, such as derivatives of fungal pyripyroneA, are under scrutiny [87].

5.5. *Diacylglycerolacyltransferases inhibitors*

Diacylglycerolacyltransferases (DGATs) are enzymes involved in adipocyte lipid accumulation and catalyzes the final step reaction of triacylglycerol formation from diacylglycerol [88]. DGAT1 belongs to the same family of proteins as the ACATs [89]. In mammals, DGAT1 is expressed in skeletal muscle, skin, intestine (ileum, colon), and testis, with lower levels of expression in liver and adipose tissue, while DGAT2 is ubiquitous with high expression levels in hepatocytes and adipocytes [90].

5.6. *Microsomal TG transfer protein inhibitors*

Microsomal TG transfer protein (MTTP) is a heterodimeric lipid transfer protein that catalyzes the transport of TG, cholesteryl ester, and phosphatidylcholine between membranes [91]. MTTP is a protein located in intestine and liver tissues where it plays a role in lipid assembly, transport, and secretion of lipoproteins, triglyceride rich chylomicrons (in enterocytes), and VLDL (in hepatocytes) [92,93]. *In vitro* studies show that MTTP catalyses the transport of molecules between phospholipid membranes and is also involved in the synthesis of nascent lipoprotein particles within the lumen and ER [94]. The inhibition of MTTP by small molecules should lead to the reduction in plasma TGs and cholesterol levels [95].

Some clinical candidates, such as CP-346086 and BMS-201038, have been shown to inhibit MTTP in both the enterocytes and in the liver [96]. Dirlotapide is an enterocyte-specific MTTP inhibitor that has recently been approved by the FDA as an anti-obesity agent [97]. The most significant side effects involve elevation of hepatic transaminases, nausea, diarrhea, gassiness, and gastrointestinal cramping [98]. Of several MTTP inhibitors, only BMS-201038, now renamed AEGR-733, is still in development [98]. Microsomal triglyceride transfer protein inhibition with lomitapide may offer a treatment option for patients who cannot tolerate statin therapy or who experience insufficient LDL-c reduction with available therapies [95].

5.7. *Squalene synthase inhibitors*

Squalene synthase, a key enzyme in the cholesterol biosynthetic pathway, occupies the first and solely committed step towards the biosynthesis of the sterol nucleus of cholesterol; hence it is an attractive target for inhibition and the development of novel and improved antihypercholesterolemic agents [99]. Squalene synthase catalyzes one of the subsequent reactions in the cholesterol biosynthetic pathway (i.e. it reductively dimerizes two farnesyl pyrophosphate molecules to form squalene) which is the first intermediate committed to cholesterol [100]. Squalene synthase inhibitors are emerging new stars in the hypolipidemic drug sky and represent a novel class of antihyperlipidemics [59]. Squalene synthase is implicated in the late step in cholesterol biosynthesis and, the squalene synthase inhibitors exerts same effect as that of 3-hydroxy-3-methylglutaryl-coenzyme A-CoA reductase inhibitors, with decreased cholesterol production and up-regulation of LDL receptors [79]. Early inhibitors such as the zaragozic acids showed significant toxicity (acidosis), but a recent compound, lapaquistat, reached Phase III clinical trials [101,102]. EP2306 and EP2302 have been shown to possess antioxidant properties both *in vitro* and *in vivo* [103] as well as to inhibit squalene synthase activity and lipid biosynthesis *in vitro* [104].

5.8. *Thyroid hormone analogues*

Thyroid hormone has been known to lower total serum cholesterol for many years in hyperthyroidism and during thyroid hormone replacement therapy for hypothyroidism [105]. This action is the result of an accelerated LDL-c clearance rate [106]. T3 increases levels of both the hepatic LDL receptor and its mRNA [107,108]. Additional thyroid hormone actions on lipid metabolism include increasing the activity of lipoprotein lipase [106,109]. More recent understanding of thyroid hormone receptors has led to the development of thyroid hormone mimetics that have selective functions and are potential therapeutic agents to lower cholesterol [110]. Several thyroid hormone analogues have been developed, but the only one with published human data is eprotirome [98], a thyroid hormone analogue containing two bromines that only interacts with the β-receptors found primarily in the liver. It does not seem to have adverse effects on heart and bone [108,111].

5.9. *Lanosterol synthase inhibitors*

Oxido-squalene-cyclase (lanosterol synthase, LSS) is the second enzyme below the farnesyl pyrophosphate branch point that has been identified as a target for novel anti-cholesterolemic drugs that could complement statins [112]. LSS is located in the ER and converts 2,3-oxidosqualeneto lanosterol, the initial four-ringed sterol intermediate in the cholesterol synthesis pathway. The 24(S),25-epoxycholesterol is a ligand of liver X receptor [113]. It also sets the template for the design of inhibitors with improved pharmacological properties for cholesterol lowering and treatment of atherosclerosis. Through the dual mechanism of LSS action (formation of lanosterol; formation of ligands for liver X

receptor), LSS inhibitors have a potential to decrease plasma levels LDL-c and to prevent cholesterol deposition within macrophages [59].

5.10. *Cholesterol metabolizing cytochrome P450: implication for cholesterol lowering*

From the family of P450s, the 7A1, 27A1 and 46A1 are the most important enzymes involved in the control of cholesterol levels in the periphery and brain [114]. CYP7A1 is an important determinant of plasma cholesterol levels and is considered as target for cholesterol lowering [115].

CYP27A1 converts cholesterol to 27-hydroxycholesterol by oxygenation reaction and this is suggested to be important reaction for cholesterol elimination from human lung macrophages and cells in arterial endothelium [116].

5.11. *AMP-activated protein kinase activator*

AMP-activated protein kinase (AMPK), a heterotrimetric energy sensing protein, which restores cellular energy balance by promoting ATP-generating pathways (e.g. FA oxidation) and inhibiting ATP-utilizing pathways (e.g. FA synthesis) [117]. AMPK system plays a major role in regulating glucose and lipid metabolism by effect on energy metabilism and long-term effect on gene expression in the liver [118]. In liver, activation of AMPK results in decreased production of plasma TG and cholesterol and enhanced FA oxidation [119,120]. WS070117 is synthetic lipid lowering agent that is approved preclinically as an effective activator of AMPK with potential capability of inhibition of *de novo* hepatic lipogenesis [121].

5.12. *Omega-3 FAs*

Omega-3 belongs to the polyunsaturated FA family (n-3 PUFA), which includes the 20-carbon eicosapentanoic acid and 22-carbon docosahexaenoic acid, which lowers the TG levels and atherogenic remnant lipoproteins [81]. These FAs are derived from marine sources, especially salmon, mackerel, sardines, and tuna [28]. Omega-3 FAs at 4 g/day usually have favorable effect in lowering TG concentration particularly in the postprandial state and their addition to statins significantly decreases TG, VLDL, and non-HDL-c levels compared with simvastatin alone [122]. Omega-3 FA inhibits expression of SREBP-1, which is involved in the synthesis of FAs [80]. Another broad variety of biological actions shown by omega-3 FAs are hypotriglyceridemia, antiaggregatory, anti-inflammatory, and antiarrythmatic responses [4]. The most common adverse events shown by omega 3 fatty acids in clinical trials are eructation, infection, dyspepsia, and flu syndrome [8].

6. Conclusion

Hyperlipidemia is a metabolic disorder characterized by HC and HTG. FHC is one of the types of hyperlipidemia with a genetic basis. In the present review we mainly focused on the new therapeutic drug targets in the treatment of hyperlipidemia. PPARs are regulators of numerous metabolic pathways; hence there is huge increase in the development and use of agonists of these receptors as therapeutics of dyslipidemia. Inhibition of CETP should lead to elevation of plasma HDL cholesterol and lowering of plasma LDL cholesterol. Ezetamibe is the only drug available today that acts by inhibition of dietary cholesterol absorption without affecting the absorption of fat-soluble vitamins, TGs and bile acids. The inhibitors of ACAT, DGAT, and MTTP, along with thyroid hormone analogue, cholesterol-metabolizing cytochrome P450, AMPK activators, and omega-3 FAs, will be the new therapeutic drug targets in treatment of hyperlipidemia. The inhibitors of certain enzymes such as squalene synthase and lanosterol synthase contribute to the reduction of hyperlipidemia.

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Review article

Negatively charged L5 as a naturally occurring atherogenic low-density lipoprotein

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ABSTRACT

Deranged metabolism of low-density lipoprotein (LDL) is considered the preeminent modifiable risk factor for atherosclerotic disease, and it is widely viewed as a chronic inflammatory disorder. Yet, the search for a circulating atherogenic LDL species continues, as the risk of coronary artery disease (CAD) cannot be measured by absolute LDL cholesterol concentrations in the plasma. Oxidized LDL (oxLDL) and small, dense LDL are associated with CAD, but neither has been retrieved from human plasma for mechanistic scrutiny. Electronegative LDL, a subclass of human plasma LDL, exhibits atherogenic properties in cultured vascular cells. L5, the most negatively charged subfraction of LDL, is an extreme form of electronegative LDL that we isolated through anion-exchange chromatography from the plasma of patients with increased cardiac risk (active smoking, hypercholesterolemia, type 2 diabetes mellitus, and metabolic syndrome). L5, which is scant in healthy normal patients, is as potent as artificially prepared oxLDL in inducing endothelial cell (EC) apoptosis by disrupting fibroblast growth factor 2 autoregulation that involves protein kinase B. Unlike oxLDL, however, L5 is not oxidized. Among subfractions L1–L5, which were separated by our chromatographic method, L1 is the most abundant and least negatively charged. It represents harmless normal LDL. Compared with L1, L5 has a greater content of total protein and triglycerides but a lesser amount of cholesteryl esters. Size exclusion chromatography and equilibrium density gradient ultracentrifugation indicated that L5 is neither smaller nor denser than L1. Negative charge on the particle surface has made L5 unrecognizable by the normal LDL receptor. Instead, L5 signals through and is internalized by lectin-like oxidized LDL receptor-1 (LOX-1), which has high affinity for negatively charged ligands. LOX-1 is also inducible by L5 but not L1. Through LOX-1, L5 disturbs homeostasis between the prosurvival and proapoptotic members of the Bcl-2 family, leading to mitochondrial destabilization. Additionally, it induces overexpression of various adhesion molecules and chemokines, thus promoting monocyte-EC adhesion, an early event during atherosclerosis development. Endothelial progenitor cells (EPCs) are important construction units for vascular repair and endothelial regeneration. Adding to the damage, L5 impairs EPC differentiation from

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mononuclear cells by inhibiting the induction of needed growth factor receptors. It also accelerates EPC senescence by suppressing the enzymatic activity of telomerase, which is essential for chromosome preservation. Thus, L5 is a naturally occurring, negatively charged but not oxidized LDL entity that is neither smaller nor denser than normal LDL but possesses a capacity for inducing a spectrum of atherogenic responses in vascular cells. Further investigation aimed at establishing its clinical relevance is warranted to confirm its atherogenic role. Subsequent efforts in L5 research will be directed toward the development of new diagnostic and treatment methods for CAD and other ischemic vascular diseases.

1. Introduction

There is no further doubt that low-density lipoprotein (LDL) abnormality is a primary etiology of atherosclerosis and associated complications, especially coronary artery disease (CAD). Under this notion and in view of the heterogeneity of LDL, investigators have searched extensively for the LDL species responsible for atherogenesis. Oxidized LDL (oxLDL) and small, dense LDL receive the greatest attention. Although it is supported by accumulating experimental evidence and the localization of oxidized lipids in lesions [1–3], it remains uncertain whether LDL oxidation is a cause of atherosclerosis [4]. One major reason for this doubt is that most experimental data have been derived from oxLDL that has been artificially prepared *in vitro*. Circulating small, dense LDL has been statistically related to atherosclerosis and CAD events [5,6]. Despite a possible correlation with CAD, neither form of LDL has been isolated from human plasma, to allow investigation of their effects in vascular cells. By contrast, interest is growing in another possible candidate: negatively charged LDL, a subclass of circulating LDL that is defined as either unoxidized or minimally oxidized yet potentially atherogenic.

2. Electronegative LDL

Hoff, Gotto, and associates [7–9] first used the term "electronegative LDL" when they noted that LDL isolated from human atherosclerotic lesions exhibited greater mobility toward the anode end in agarose gel electrophoresis than LDL isolated from normal plasma. Using fast protein liquid chromatography (FPLC) equipped with an ion-exchange column, Avogaro and colleagues [10] first divided human plasma LDL dichotomously into electropositive LDL(+) and electronegative LDL(−) in 1988. They were also the first to report that LDL(−) particles were "stickier" to one another and more toxic to vascular cells than their LDL(+) counterparts [10]. Since then, several groups have described the chemical composition and functional traits of LDL(−), which they isolated by using a similar protocol [11–26]. Among these investigators, Sánchez-Quesada and associates [19,20] have reported on a wide range of chemical and biologic features of LDL(−). In a 2004 review, they summarized that in cultured vascular endothelial cells (ECs), LDL(−) induces the production of chemokines, such as interleukin 8 (IL-8) and monocyte chemotactic protein 1, and increases tumor necrosis factor alpha (TNF-α)-induced production of vascular cell adhesion molecule 1 (VCAM-1). Most recently, this group reported that phospholipase C-like activity may play a role in higher aggregability of LDL(−) [25], the phenomenon originally noted by Avogaro [10].

3. L5 and negatively charged LDL

Using a different protocol, we used anion-exchange chromatography to divide plasma LDL from patients with familial hypercholesterolemia heterogeneity and those with moderately increased LDL cholesterol (LDL-C) into increasingly negatively charged subfractions, L1-L5 [27,28]. Strictly speaking, in chemistry, the term "electronegativity" is defined as the tendency of an atom to attract a bonding pair of electrons. On the Pauling scale, fluorine (the most electronegative element) is assigned a value of 4.0; values range down to 0.7, in the case of cesium and francium (the least electronegative elements) [29]. L5 is only relatively more negative in surface charge than other subfractions, and the complexity of an LDL particle prevents accurate calculation of its electronegativity; we decided to define L5 as the most negatively charged LDL subfraction. This allows us to avoid inaccurately implying that L4–L1 are positively charged LDL particles. Among all subfractions, L5 is the only subfraction capable of inducing apoptosis in cultured vascular ECs (Fig. 1).

4. Chemical basis of L5 negative charge and its biologic implications

We previously reported that L5 accounts for approximately 2% of total plasma LDL in asymptomatic patients with type 2 diabetes mellitus (DM) [30]. L1, the least negatively charged subfraction, represents a majority (> 85%) of total LDL. The content of both protein and triglyceride (TG) increased progressively in the direction of L1 to L5, whereas that of cholesteryl esters decreased. The content of phospholipids and free cholesterol was largely the same among all subfractions [30]. These findings concur with those reported for LDL(−) [12]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed higher total protein content in L5 particles, which is partially explained by the inclusion of apolipoprotein (apo)-AI, apoE, and apoCIII, as well as apoB-100, which is the sole apolipoprotein of L1 [30]. Additional experiments with L1–L5 isolated from hypercholesterolemic human plasma yielded

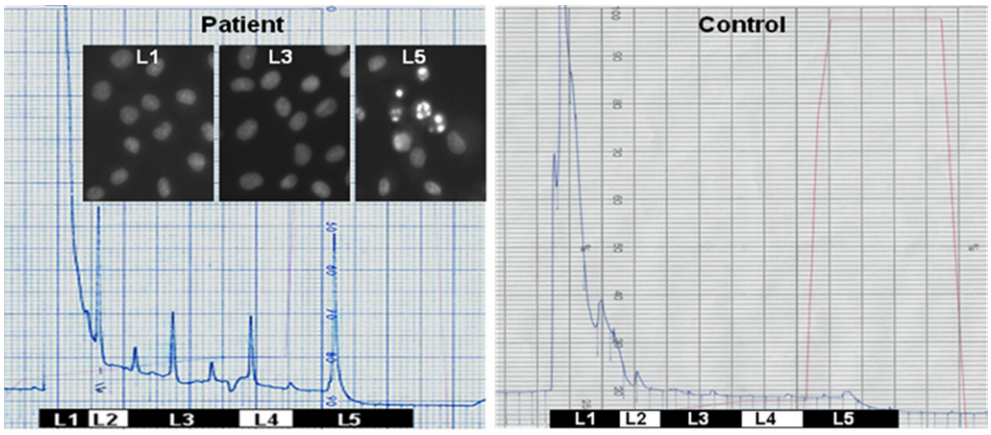

Fig. 1 – Isolation of L5 and other LDL subfractions by FPLC. L5 is present in patients with elevated LDL-C (>160 mg/dL) but not in nondiabetic, normolipidemic healthy subjects. L5 is the only subfraction that induces marked EC apoptosis, as demonstrated by nuclear condensation and fragmentation. C = cholesterol; EC = endothelial cell; FPLC = fast protein liquid chromatography; LDL = low-density lipoprotein.

similar results; Fig. 2 shows a representative SDS-PAGE gel. Fig. 3 illustrates similarities and differences between L5 and L1. Because L5 contains twice the amount of TG than L1, it can be regarded as a TG-rich LDL by comparison. Compatible with our findings, Bancells and colleagues [26] demonstrated recently by proteomics analysis that the amounts of apoE, apoAI, apoC-III, apoAII, apoD, apoF, and apoJ are higher in LDL(–) than in LDL(+). In our latest experiments with liquid chromatography/mass spectrometry (LC/MS^E), we quantitated in detail all apolipoproteins and other low molecular weight proteins associated with L5. Selective association of these molecules with low isoelectric points in L5, but not L1, in part explains the relatively high negative charge on the surface of L5 (unpublished data), which may contribute to the altered affinity of L5 for the normal LDL receptor (LDLR). The chemically oriented shift of L5's affinity from LDLR to other receptors constitutes the basis of its unique pathologic functionality.

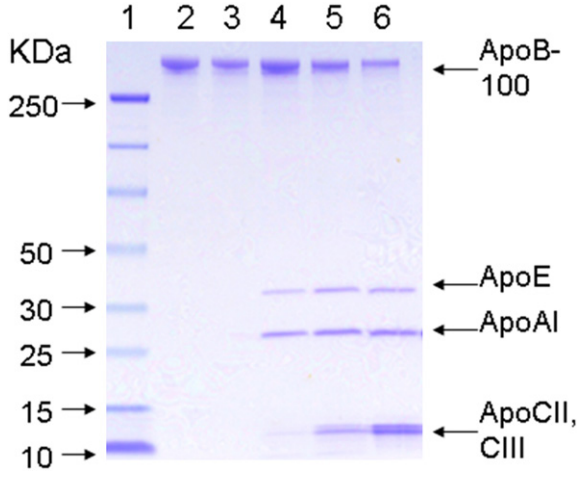

Fig. 2 – Representative 4-20% SDS-PAGE gel that shows the apolipoprotein distribution in L1–L5 from hypercholesterolemic human plasma. Protein profiles are shown for L1-L5 (from left to right: Lanes 2-6); apoB-100 is the sole protein in L1, whereas L5 also contains apoE, apoAI, and apoCII/CIII. apo = apolipoprotein; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

5. L5 vs. oxLDL and small, dense LDL

Although L5 and copper-oxidized LDL are equally potent in suppressing the transcription of fibroblast growth factor 2 (FGF2) and inducing apoptosis in vascular ECs [28,31], they differ widely in their chemical and physical characteristics. The production of thiobarbituric acid-reactive substances (TBARS) is often used as a measure of oxidative lipid modification. After copper and oxygen exposure for 24 hours, the TBARS value of oxLDL often reaches the high concentration of 18–22 nmol/mg protein. In contrast, the TBARS value of L5 is mostly less than 1 nmol/mg protein, which is no different than that of L1–L4 [28]; this is in accordance with observations made in LDL(–) [11,20,22]. Various artificial oxidation methods increase the negative charge of normal LDL and turn it into electronegative LDL [17,32]. In preliminary experiments, copper oxidation of L1 yielded ox-L1, which exhibited increased electrophoretic mobility on agarose gel, as well as proapoptotic effects, as seen with non oxidized L5 (data not shown). However, neither oxLDL nor ox-L1 has the same chemical composition as naturally occurring L5. Therefore, L5 is a nonoxidized, naturally occurring atherogenic LDL.

L5 is a subfraction of plasma LDL, which is defined by density. The high TG content and the association with apoCIII, apoE, and apoAI suggest L5's close resemblance to remnant-like particle cholesterol [33,34]. Size exclusion chromatography and equilibrium density gradient ultracentrifugation showed that L5 is no different from L1–L4 in either particle size or density [35]. This suggests that L5 is neither smaller nor denser than other particles in the same lipoprotein class defined by density. Small, dense LDL is defined by particle size on gradient gel electrophoresis or nuclear magnetic resonance (NMR) [36]. Small, dense LDL is considered atherogenic chiefly because it has a greater propensity for oxidation, and it may be concluded that it is an etiologic agent in atherosclerosis. The association of

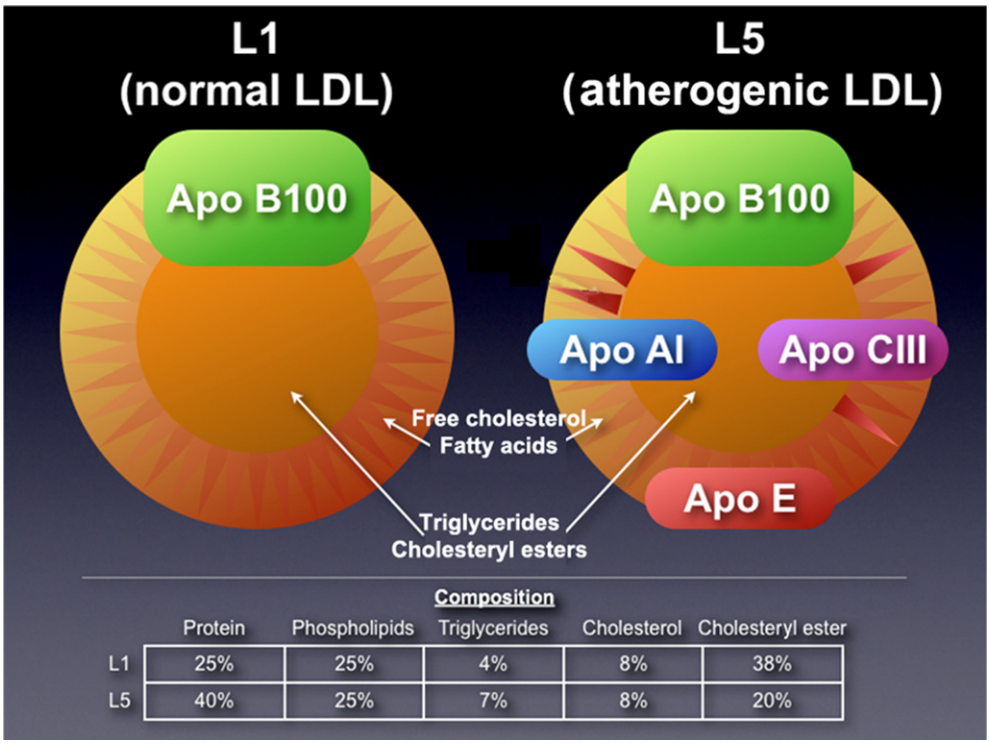

Fig. 3 – Schematic comparison between L1 and L5 isolated from hypercholesterolemic human plasma. L5 and L1 differ in their lipid and protein compositions. LDL = low-density lipoprotein; apo = apolipoprotein.

LDL particle size with cardiovascular diseases has been tested for magnitude and independence in many studies, e.g., clinical intervention trials, and cross-sectional and prospective epidemiologic studies. Nearly all show a significant univariate association of small, dense LDL with increased CAD risk, but LDL size is seldom a significant and independent predictor of CAD risk after multivariate adjustment for confounding variables, in particular, plasma TG and high-density lipoprotein cholesterol (HDL-C) concentration. Hence, it may be that the increased risk associated with smaller LDL size in univariate analyses arises from broader pathophysiologic causes, of which small, dense LDL is a part, rather than a reflection, of intrinsic increased atherogenic potential. Thus, a clear causal relationship between small, dense LDL and increased cardiovascular risk has not been proven [37]. This further accentuates the importance of L5, which is not a small, dense LDL, in atherogenesis.

6. Receptors for and active components of L5

Our data suggest that at least two receptors, platelet activating factor (PAF) receptor (PAFR) and lectin-like oxidized LDL receptor-1 (LOX-1), are involved in transducing L5-elicited signaling of ECs and endothelial progenitor cells (EPCs) [31,38,39]. Protein kinase B (Akt) exerts multiple prosurvival and vasomotor effects by activating downstream targets, such as endothelial nitric oxide synthase (eNOS), after its own activation by phosphatidylinositol 3-kinase (PI3K) [40,41]. L5 inhibits EC proliferation and induces EC apoptosis, disrupting FGF2 autoregulation via an FGF2-PI3K-Akt loop [31]. These effects are significantly attenuated by pharmacological blockage of PAFR or by inhibiting G protein incorporation into PAFR, a G protein-coupled receptor [28,31,42].

LOX-1, though originally cloned against copper-oxLDL, has a strong affinity for negatively charged particles through an electrostatic interaction [43,44]. We reported that, unlike L1, L5 is not recognized by LDLR on the plasma membrane of ECs and EPCs, but it is internalized by LOX-1 in a competitive manner against artificially prepared oxLDL [31,38]. Preliminary experiments suggest that the low pI values of non–apoB-100 protein molecules contribute to the switch in affinity from normal LDLR to LOX-1 (unpublished data), which is also inducible by L5 but not L1 [31,38].

The active components of L5 are not yet fully identified, but our current data suggest that they reside on lipids that accumulate in L5 particles. The total lipid extract derived from L5 is as efficient as L5 in inducing intracellular calcium transient in polymorphonuclear neutrophils (PMNs) [28]. Pretreatment of L5 with PAF acetylhydrolase to degrade PAF and PAF-like lipids via hydrolysis of *sn*-2 residues removes L5's capacity to downregulate FGF2 and induce EC apoptosis. Also, the lipid extract of degraded L5 fails to elicit calcium transient in PMNs, which is readily restored by adding exogenous PAF [28]. Findings suggest that protein configuration is important for receptor recognition but that lipid components, especially certain phospholipids, are responsible for signaling.

7. Evidence of L5's atherogenicity *in vitro*

Apoptosis of the vascular endothelium contributes to increased transendothelial permeability [45,46]; released microparticles

enhance tissue factor expression and thus provoke coagulation [47,48]. Evidence that L5 induces EC apoptosis in a concentration- and time-dependent manner indicates L5's involvement in both early and late stages of atherothrombosis [28,30,31,35,39]. In addition to EC apoptosis, L5 induces monocyte-EC adhesion, another early event in atherosclerosis development [27,28]. Cell-to-cell adhesion is promoted by EC secretion of adhesion molecules, including VCAM-1, IL-8, and CXC chemokines [49], which is in agreement with what has been described for LDL(−) [20,50,51].

FGF2 functions by activating downstream kinases and effectors, including Akt, Bcl-2, Bad, Bax, Bcl-xL, and eNOS [52]. These effectors are major regulators of mitochondrial function and structural integrity [53]. We recently demonstrated that L5 suppresses the expression of the mitochondria-stabilizing and prosurvival effectors Bcl-2, Bcl-xL, and eNOS, as well as eNOS phosphorylation, and upregulates the pro-apoptotic effectors Bax, Bad, and TNF-α in vascular ECs through LOX-1 [39]. The L5-initiated signaling that leads to endothelial dysfunction and atherogenesis is summarized in the schematic illustration seen in Fig. 4.

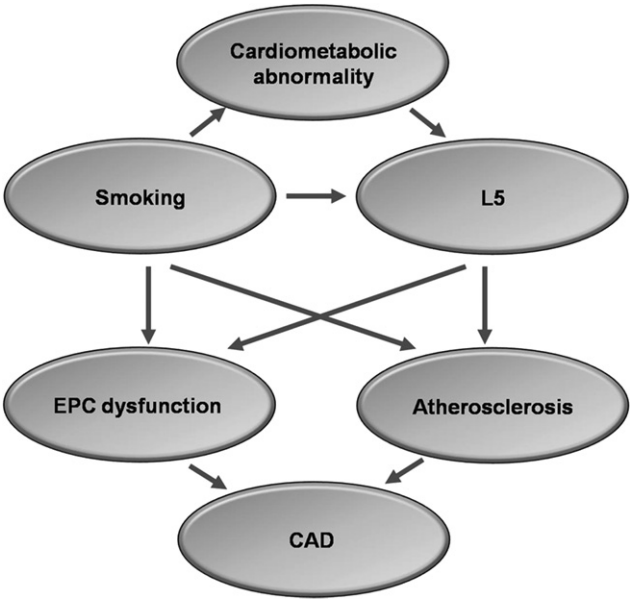

Fig. 5 – Risk factors for L5 formation and the consequent effects on cardiovascular health. Cardiometabolic abnormalities include hypercholesterolemia, type 2 diabetes mellitus, and metabolic syndrome. CAD = coronary artery disease; EPC = endothelial progenitor cell.

8. L5 and EPC physiology

Evidence suggests that bone marrow-derived EPCs play a key role in endothelial regeneration, as well as in vasculogenesis

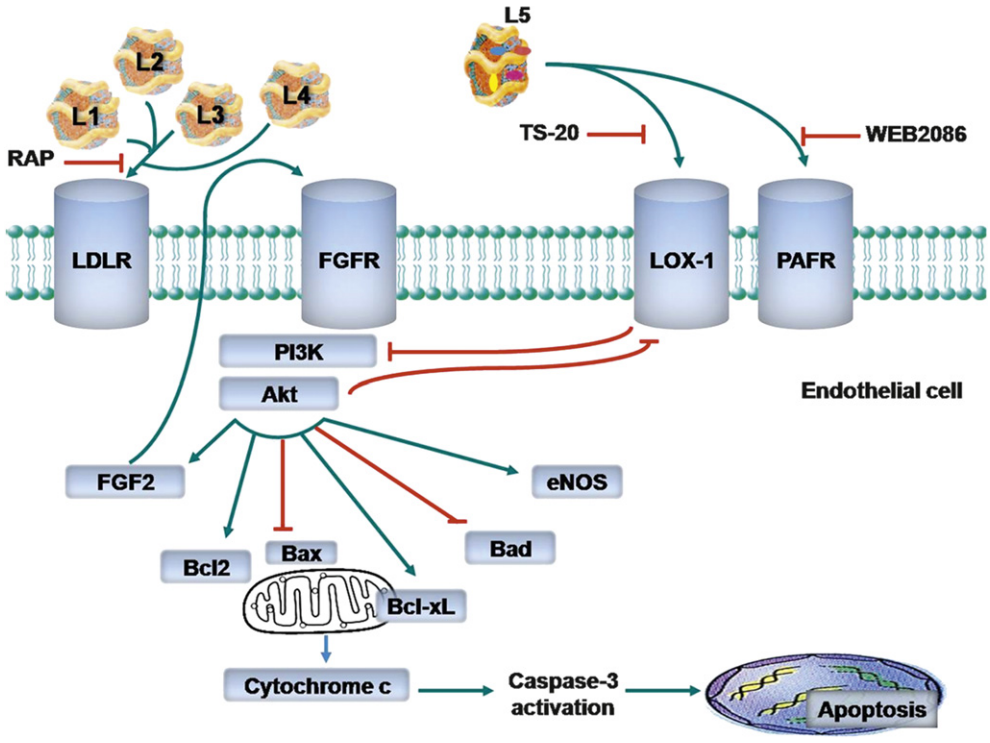

Fig. 4 – Schematic summary of L5 signaling through LOX-1 in vascular ECs. L5 signals and is internalized through LOX-1, whereas L1–L4 are endocytosed via LDLR. Green arrows indicate stimulation; blue arrows indicate release from mitochondria; red lines with end bars indicate inhibition. RAP = the LDLR inhibitor receptor-associated protein; TS-20 = LOX-1 neutralizing antibody; EC = endothelial cell; LDL = low-density lipoprotein; LDLR = LDL receptor; LOX-1 = lectin-like oxidized LDL receptor-1; FGFR = fibroblast growth factor receptor; PAFR = platelet-activating factor receptor; WEB2086 = thieno-triazolodiazepine, an antagonist of PAF; PI3K = phosphatidylinositol 3-kinase; Akt = protein kinase B; FGF2 = fibroblast growth factor 2; Bcl-2 = B-cell lymphoma 2; Bax = Bcl-2-associated X protein; Bcl-xL = B-cell lymphoma extra large; Bad = Bcl-2-associated death promoter; eNOS = endothelial nitric oxide synthase.

[54–56]. EPCs are reduced in number and/or functional activity in the presence of traditional and emerging major risk factors, whether separately or as clusters [57]. Reported correlated risk factors include aging [58], subclinical and clinical atherothrombotic disease [59,60], types 1 and 2 DM [61,62], hypercholesterolemia [63], smoking [57], and metabolic syndrome [64]. One common trait in people with these risk factors is the percentage increase of L5 in their plasma LDL [27,28,30,35,38,65,66]. At subapoptotic concentrations, L5 inhibited vascular endothelial growth factor (VEGF)-induced differentiation of human circulating monocytes into EPCs; this is achieved by suppression of Akt-mediated induction of VEGF kinase insert domain-containing receptor and other endothelial cell markers [38]. The impairment of mitogenic activity in early EPCs by abnormal LDL can accelerate EPC senescence [67]. Cellular senescence is critically influenced by telomerase, which elongates telomeres and thereby counteracts the telomere length reduction induced by each cell division [68,69]. In our setting, L5 accelerates EPC senescence by inhibiting telomerase activity [38], thus severely compromising the regenerative capacity of progenitor cells.

9. Conclusions and perspectives

Based on our findings and a review of the literature, we conclude that L5 is a non oxidized, naturally occurring atherogenic LDL that is not smaller or denser than other LDL subfractions. In preliminary experiments, repeated injection of human L5 into apoE knockout mice induced atherosclerotic changes in the aorta, and addition of L5 into organ chambers attenuated the endothelium-dependent relaxation of aortas that were removed from rats (data not shown). These *in vivo* and *ex vivo* observations support a role for L5 in endothelial dysfunction and atherosclerosis formation. Our recent data also suggest that (a) L5 is increased in patients undergoing acute coronary events and that (b) intracoronary thrombi contain tissue that exhibits strong LOX-1 expression. Further study is needed to confirm the role of L5 in atherothrombotic development in animals and in humans. Fig. 5 summarizes the links between risk factors that favor L5 formation and the consequences of L5 accumulation. Our ongoing studies aim to develop both diagnostic and therapeutic methods for the early detection and effective treatment of L5-mediated vascular disease.

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Review article

Androgen and androgen receptor signals jamming monocyte/macrophage functions in premalignant phase of livers

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ABSTRACT

There is a widely discussed concept that chronic inflammation and repeated damage-repair cycles result in malignant transformation of the liver. Kupffer cells are the major host defending macrophages that reside in the liver. They are also considered as critical in episodes of cancer immune surveillance that wipe out liver malignancies, yet, turn into cancer assisting cells in certain conditions. Monocyte/macrophage population and cytokine profile hierarchization in hepatocellular carcinoma (HCC) has increased the curiosity to search for macrophage modulators. Androgenic signals [androgen/androgen receptors (A/AR)] play an important role in liver function and disease progression. Basic and clinical studies have revealed that A/AR might play a biphasic function in HCC progression. Whether A/AR work with TAM to further manipulate HCC progression is of great interest. In this review article, we will focus on the interaction of hepatocytes and monocytes/macrophages in preneoplastic/ inflammatory liver diseases, underlining A/AR actions.

1. Introduction

1.1. *Androgen/androgen receptor (A/AR) roles in hepatic host immunity*

The liver is the largest visceral organ and is responsible for systemic homeostasis such as blood glucose, lipid and protein metabolism and the clearance of xenobiotics [1]. There are several different cell types found in a liver unit: hepatocytes, Ito cells (lipocyte), Kupffer cells (monocytes), and oval/stellate cells (fibroblast) [1]. The hepatic sinusoids are unique structures that are lined with a thin discontinuous endothelium. Kupffer cells are part of this thin line and are responsible for scavenging hepatic debris for the hepatic host immune response. Liver is under a constant immunological challenge, and the immunological response is dominated by innate immunological components including macrophages, dendritic cells, natural killer cells, natural killer T cells, complement

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components, acute phase proteins, and chemokines [2]. The immune surveillance function (tolerant to daily food absorption to the liver) is largely executed within hepatic reticuloendothelial cells, which are largely composed of Kupffer cells. The cell surface receptors, e.g., TLR, complement receptor and Fc-receptors on Kupffer cells, are known to be largely responsible for sensing innate immunological responses [2,3]. However, little is known in diet-induced liver damage.

During acute illnesses, acute innate immunity could be activated by the activation of macrophages, and by producing certain acute phase proteins. Macrophages and lymphocytes could secrete inflammatory cytokines such as tumor necrosis factor-alpha (TNFα), interleukin-1 (IL-1), and interleukin-6 (IL-6) when antigens are present in the organs. Interacting with complement pathways, the complex could bring microbes to phagocytes, cause macrophage cytolysis, and chemotactically attract phagocytes to the infected area. Inflammatory cytokines travel through the blood and stimulate hepatocytes in the liver to synthesize and secrete acute phase proteins. This response provides an early defense and enables the body to recognize foreign substances early on in the infection process, prior to the full activation and implementation of the immune responses [2,3].

Using an AR knockout animal model, Lin et al explored the potential roles of A/AR signals in food-induced hepatosteatosis and the relative metabolic syndrome in the mouse [4]. In this report, Lin et al pointed out that AR could act as protein-tyrosine phosphotase 1B (PTP1B) suppressors, which suppress insulin signaling and inhibit β-oxidation of lipids in hepatocytes. However, as previously described, Kupffer cells are host immune confounders against metabolic and chronic steatosis of the liver. Introducing the 5a-reductase inhibitor, 4-hydroxyandrostenedione (4-OHA), suppresses testosterone conversion to 5a-dihydrotestosterone (DHT) in mice and could reduce Kupffer cells cytokine production, but not splenic macrophages [5]. Their studies provide some evidence to suggest that A/AR might also play a role in the overindulgent stress in which gate-keeping by host immune defense in the liver.

1.2. *A/AR roles in the hepatic damage/regenerating state*

Liver regeneration is the homeostatic machinery to recover the liver structure and function after physical or chemical hazards [6]. Distinguishable from other regenerative organs (skin, bone marrow), the liver regenerates from large populations of mature cells, and not from small amounts of stem/progenitor cells [6,7]. Several distinct cells, such as hepatocytes (the main functional cells of the liver), cholangiocytes (biliary epithelial cells), fenestrated endothelial cells (gates between blood fluid and hepatocytes), Kupffer cells (liver macrophages), and Ito cells (stellate cells; extracellular matrix secretion, growth factors production, and vitamin A storage) [6] are involved in the healing process. Among them, hepatocytes are the primary and most important initiators during the whole liver regeneration process.

There are several animal models using rodents for studying liver regeneration. The most common is the removal of two-thirds of the liver (partial hepatectomy; PHx) to induce liver regeneration. Another is the injection of the hepatic toxin, CCl4, to facilitate hepatic proliferation. Results from these animal studies suggested that proliferation within the hepatic parenchyma starts from the portal triad to the pericentral area within 48 hours. Several immediate early response genes, such as NFκB and STAT3, could be activated rapidly (within 60 minutes) after PHx [8,9]. Other factors involved in liver regeneration after PHx are the growth factors and cytokines, such as hepatocyte growth factor (HGF), tumor necrosis factor alpha (TNFα), interleukine-6 (IL-6), epidermal growth factor and transforming growth factor alpha [10]. Nuclear receptor signals, such as thyroid hormone [11], retinoid acids [12], and glucocorticoid [13] as well as estrogens and androgens, also play important roles in liver regeneration.

In general, the liver regenerative response is better in females than in males [14], suggesting that A/AR signals may play a negative role in regenerating the liver. The decreased serum androgen levels, accompanied by down-regulation of AR protein in the liver during liver regeneration, also implicates the negative role of A/AR signals [15,16]. In addition, Kan et al, found that using flutamide (an androgen action antagonist) can protect trauma-hemorrhage-induced liver injury through reducing the systemic inflammatory response [17]. Published by same group, Schneider et al found that trauma and hemorrhage-shock induced TNFα and IL-6 production is by Kupffer cells and splenic macrophages [5]. In other words, in the normal liver, A/AR might balance the other stimulating signals, such as growth factors or prolactin [18–21]. Once the liver has been damaged, the homeostasis machinery tends to suppress negative factors, i.e., A/AR signals, to facilitate liver regeneration.

However, other reports also suggested that A/AR signals might play a small role in the liver regeneration. For example, the antiandrogen, cimetidine, showed little effects on PHx rat liver [22], and administration of tamoxifen in male hepatectomized rats resulted in increased AR activity, yet had little effect on liver regeneration [18]. Similarly, the addition of the antiandrogen, flutamide, resulted in initial overexpression of AR, yet had little effect on liver regeneration in PHx rats [23].

Kahn et al [22] also demonstrated that in the rat liver, hepatic injury with a portacaval shunt results in minor effects on AR activity, and partial clamp of the portal vein or clamp of the hepatic artery yields little effect on AR activity. However, using CCl4 toxication in the liver, Smirnova et al [19,20] and Shchelkunova et al [25] found that A/AR signals might indirectly facilitate liver regeneration through modulation of a specific protein, unusual estrogen-binding protein (UEBP), in the hepatocytes that can increase the uptake of estrogen.

The above contradictory effects (suppression vs. promotion vs. little influence) of A/AR signals using either androgen administration or surgical castration to test the liver regeneration ability in different animal models (PHx or CCl4 toxication) implied the complexity of A/AR signals during the liver regeneration process. There is no perfect explanation for the contradictory effects of A/AR signals between liver regeneration models. Whether the differential secretion of growth factors or cytokines that were induced by surgical hepatectomy, or chemical toxication in different animal models, results in such diverse A/AR responses, is an interesting question for further study.

1.3. A/AR signals and hepatitis B virus (HBV) virus antigen expression and replication

The evidence that A/AR signals influence HBV replication was based on HBV transgenic mice studies. Although HBV virions, HBsAg and HBeAg, can be detected in HBV transgenic mice, the mice did not exhibit pathological changes because of the immune tolerance. The high HBV DNA amount is associated with the occurrence of human HCC, so it is important to know how A/AR signals affect HBV antigen expression and replication in mice. It has been found that serum HBsAg concentration is higher in male than in female HBV transgenic mice. Castration of male mice could eliminate this sexual dimorphism, while supplementation with testosterone could restore the difference [26–28]. Furthermore, in order to determine the effects of AR onthe HBV virus, Breidbart et al [26], using testicular feminization mutation (Tfm) mice, in which 90% of AR was reduced, found that the serum HBsAg concentration is higher in wild type mice compared with XYTfm mice. Except for direct A/AR signals on regulating HBV antigen expression [29], more evidence is required for the effect of A/AR signals in HBV-related HCC. A previous bottleneck of HBV-HCC studies was due to the lack of proper animal models in which HCC spontaneously develops from hepatitis B. Woodchuck hepatitis virus (WHV) is similar to the human HBV, both in structure and replicative viral life cycle. WHV infection can cause acute and chronic hepatitis. Chronic WHV infection in woodchuck will develop into HCC within the first 2 to 4 years [30]. Therefore, the WHV model might allow us to know more about the effects of A/AR in the progression of HBV infection and the related liver disease. Recent studies showed that androgenic signals promote HBV viral replication through direct regulation of HBV replication [31–34]. Furthermore, Wu et al, found that HBV transgenic mice, supplemented with a subminimal dose of carcinogen, can induce spontaneous HCC development, while knockout hepatic AR could reduce HBV-related hepatocarcinogenesis through direct regulation of HBV virus replication [31].

1.4. A/AR signals in cirrhotic livers

Cirrhosis is the pathological feature that can be observed in diverse liver diseases, generally arising from chronic liver injury. During the injury healing process, the liver could develop fibrotic lesions that may lead to the loss of normal hepatic function, impaired liver regeneration, aberrant polarity for cells to proliferate, and obstruction of the portal system's ability to secrete bile acid. Based on the population epidemiology, liver cirrhosis can be classified into three categories: (1) alcoholic-related cirrhosis (ARC); (2) virus-induced hepatitic cirrhosis; and (3) non- alcoholic cirrhosis.

The linkage of male hypogonadism and hypotestosteronemia with ARC victims [35] suggests that A/AR signals might play some role in the ARC. Reduced testicular size and the clinical features of inadequate testicular function are manifested as clinical hypogonadism in cirrhotic men. Between 50% and 75% of cirrhotic men have both macroscopic and histological testicular atrophy. Associated with this, 80%–90% of cirrhotic men are impotent, and seminal fluid characteristics are grossly abnormal in the small minority of patients who are able to produce an ejaculation [35]. Furthermore, cirrhotic men have a decreased incidence of benign prostatic hypertrophy, and gynecomastia could also be found in about 40% of cirrhotic men [24].

Testosterone administration can improve both hypogonadism and gynecomastia syndrome effectively, however, it may have little effect in improving cirrhosis [36]. This suggests that the decrease of testosterone, due to irreversible damage of the liver, may be a consequence of cirrhosis. Interestingly, Kley [37] found that administration of testosterone to male ARC might yield some improvement. In agreement with this, Thole et al [38] also reported that administration of steroidal or non-steroidal antiandrogens, such as cimetidine or flutamide, might lead to cirrhosis [24], although the potential toxicity of these antiandrogens might also contribute to such cirrhosis.

In contrast, Gluud C et al [39,40] found that oral testosterone treatment yielded little change in the liver pathogenesis of ARC men, even when such treatment could significantly reduce the prevalence of gynecomastia. Similarly, a large population in a randomized clinical trial also found androgens, such as testosterone and oxandrolone administration could not improve ARC patients.

2. Concluding remark: Illustrating the relations within Kupffer cells, A/AR and hepatocarcinogenesis

Recent reports on A/AR signals in hepatocarcinogenesis have gained advances regarding carcinogen- and HBV-related liver malignancy [31,41,42]. However, the bimodal function of A/AR has also been described in HCC progression [43]. Although there is little information regarding A/AR signals in the immune surveillance of hepatocarcinogenesis, A/AR signals did have documented in prostatic malignancy in terms of innate and adaptive immunities. Published by Lai et al, in the immune cell-specific AR knockout mice described, A/AR is pivotal for both innate and adaptive immune regulations [50,51].

It has been reported that innate immunity plays a pivotal role in hepatocarcinogenesis. By removing NFκB signals in the hepatocytes, the mouse became resistant to carcinogen-induced HCC [44]. This result clearly demonstrated the linkage of innate immunity to HCC. The Kupffer cells population is not increased in the cancer lesion. The Kupffer cell number may even be decreased [45]. These conflicting findings could lead to the conclusion that innate immunity might be an important factor, however, the innate immunity does not necessarily require invasive macrophages into the tumor lesion. Previous studies, in both humans and rodents, have shown that tumor necrosis factor alpha (TNFα) is an important mediator of liver injury [46–48]. Although many types of cells in the liver are capable of producing TNFα, Kupffer cells are thought to be the main hepatic source of TNFα. They also produce factors such as IL-12 and IL-18 and interferon gamma, which enhance TNF activity, as well as those that inhibit TNF-α, such as IL-10 [49]. The role of A/AR in the whole process of HCC development and progression is still a mystery. Whether A/AR and TAM participate in the hepatocarcinogenesis process is yet to be explored.

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Review article

Incorporating behavioral research to examine the relationship between betel quid chewing and oral cancer in Taiwan

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ABSTRACT

Cancer of the oral cavity is one of the most commonly diagnosed cancers and one of the leading causes of death among men in Taiwan. Extensive research findings have linked betel quid chewing with oral cancer and precancerous conditions. To date, no pharmacological or behavioral treatments exist for betel quid cessation. This paper discusses the potential benefits of applying behavioral research to better understand why betel quid chewers consume betel quid. Specifically, it discusses using behavioral research methods to examine betel quid chewing initiation, dependence, motivation, and withdrawal. Better understanding of these different aspects of betel quid chewing is likely to aid researchers in developing treatment programs.

1. Introduction

Cancer of the oral cavity is one of the fastest growing cancers among men in Taiwan. Between 1979 and 2006, the age-adjusted incidence for oral cancer among men increased seven times, from 5.04 per 100,000 to 35.88 per 100,000, an increase that is greater than that of the other nine most common cancers among Taiwanese men [1]. In 2006, cancer of the oral cavity was the fourth most commonly diagnosed cancer and the fourth leading cause of cancer death among men in Taiwan.

The International Agency for Research on Cancer has identified tobacco smoking and betel quid (BQ) chewing, with or without tobacco, as Group 1 carcinogens to humans [2]. Both habits have been found to be significantly related to oral cancer among men in Taiwan. In this paper, we review current research on BQ use in Taiwan and the evidence regarding the link between BQ chewing and smoking and oral cancer among Taiwanese men. We also discuss the need for cessation of BQ chewing and smoking as a form of primary cancer prevention and as reduction of risk for additional cancer following cancer diagnosis and treatment.

2. BQ chewing in Taiwan

It is estimated that some 600 million people use BQ worldwide [2]. BQ chewing is popular in many Asian countries. Three types of quid, *lao-hwa* quid, BQ, and stem quid, are typically found in Taiwan [2]. *Lao-hwa* quid and stem quid are prepared by adding, respectively, a piece of inflorescence of *Piper betle* L.

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with red lime paste or a piece of *Piper betle* L. stem to an unripe acreca nut. A BQ is made by wrapping a split unripe areca nut with slaked lime paste with a piece of betel leaf. In contrast, the practice in many southeast Asian countries, tobacco is never added to BQ in Taiwan.

The 2005 Taiwan National Health Interview Survey [3] found that, among the 16,542 adult respondents (age 18–64 years), 9.2% reported that they were current BQ chewers, 4.5% indicated that they had quit in the last 6 months, 7.2% indicated that they had tried BQ but were not regular users, and 79.1% responded that they had never used BQ. Of the current chewers, 42% chewed BQ daily, 16% chewed BQ on 3–5 days/week, and 22% used BQ on 1–2 days/week, and 20% chewed <4 days/month. Most chewers were men. Although 17% of all men living in Taiwan reported chewing BQ, <1% of all women reported regular use of BQ. Several other studies have confirmed this gender disparity in BQ chewing among adults [4–6] and adolescents [7,8]. The National Health Interview Survey also found that the majority of chewers were aged 35–44 years, lived in rural and less urbanized areas, and reported a family income of lower than NT$30,000. Furthermore, individuals who completed junior high school or less in education and those who worked in nonprofessional, blue-collar occupations were more likely to chew BQ [9].

Due to historical and cultural factors, BQ chewing is especially widespread among ethnic aborigines, who make up approximately 2% of the population in Taiwan [10]. A stratified random survey that examined 7144 individuals in over 50 aboriginal communities found that 53% of male and 38% of female aborigines reported current or former use of BQ [10]. Three other large-scale surveys that sampled both aborigines and nonaborigines have also confirmed a higher rate of BQ chewing among aborigines [5,9,11]. The prevalence was estimated to be as high as 61% for men and 79% for women in a study that surveyed BQ chewing in a single aboriginal community in southern Taiwan [12]. Thus, the gender disparity that is found in the Taiwanese general population does not exist and may even be reversed among the ethnic aboriginal population.

Cigarette smoking and alcohol drinking have been found to be significantly associated with BQ use [5,8–10]. For example, Wen et al [13] analyzed data collected in the 2001 National Health Interview Survey in Taiwan and found that 93% of BQ chewers were current cigarettes smokers. It was also estimated that 50–67% of betel quit chewers consumed alcohol regularly [9,10].

We identified three surveys that examined unaided BQ cessation [9,10,14]. One study, which classified chewers as successful quitters if they had not chewed any BQ in the last 12 months, reported quit rates of 8.2% in male aborigines and 6.7% in female aborigines [10]. Using a less stringent criterion in which successful quitters were defined as chewers who had not chewed any BQ in the last 6 months, Lai et al [14] found that 49% of the Chinese (i.e., nonaborigine) chewers in their study had quit successfully. Similarly, Yap et al [9] reported that 28.6% of male nonaborigine chewers and 15.7% of female nonaborigine chewers had quit chewing in the last 6 months. Aborigine chewers had less success in quitting, with 7.8% of male and 9.8% of female chewers stopping chewing for at least 6 months. No information on relapse was discussed in any of the three studies.

Several factors, including gender, alcohol use, and level of education, have been found to be predictive of BQ cessation. Regarding gender, while one study found that male chewers were significantly more likely than female chewers to quit [odds ratio (OR) = 4.22, 95% confidence interval (CI) = 3.74–4.77] [9], another study found that although men quit at a higher rate than women, the odds ratio only approached significance (OR = 1.25, 95% CI = 0.95–1.64) [10]. These studies also found that those who drank alcohol and chewers who completed less education were significantly less likely to quit [9,10,14]. Younger chewers were more successful than older chewers in quitting [9,10]. The findings on the association between smoking and BQ cessation were mixed. One study found that chewers who did not smoke were more successful in quitting than those who did [9], whereas another failed to find significant difference in quitting success between smoking and non-smoking chewers [10].

3. BQ chewing, cigarette smoking, and cancer

A high incidence of oral cancer has been observed in countries where tobacco was routinely added to BQ [2]. In Taiwan, where tobacco is never added to BQ, numerous studies have found that BQ chewing, by itself or in combination with smoking, is significantly associated with precancerous oral lesions, cancer, and cancer death.

3.1. *Oral submucous fibrosis and oral leukoplakia*

Oral mucosal lesions such as oral submucous fibrosis (OSF) and oral leukoplakia (OL) are risk factors for and have been found to transform malignantly to oral cancer [15,16]. Two case-control studies have investigated the effects of BQ chewing and cigarette smoking on OSF and OL [17,18]. One study that examined 62 histologically diagnosed OSF patients and 62 matched controls found that BQ chewers were 4.51 times (95% CI = 1.20–16.94) more likely than nonchewers to have OSF [18]. Individuals with both smoking and chewing habits had even higher risk of OSF (OR = 8.68, 95% CI = 1.87–40.23). A significant dose response effect was found, with chewers who used between 10 and 29 quids/day having a 4.55-fold (95% CI = 1.16–17.84) increase in risk and chewers who used 30 or more quids/day having a 10.34-fold (95% CI = 2.39–44.73) increase in risk for developing OSF compared to matched controls.

In another study that included 219 patients with histologically confirmed OL or OSF and 876 community controls, the risk for developing OL was 22.3 (95% CI = 11.3–43.8) times higher for current chewers than never chewers and the risk for developing OSF was 40.7 times (95% CI = 16.0–103.7) higher for current chewers than never chewers [17]. Significant dose response effects were also found for the development of both OL and OSF. A synergistic effect of BQ chewing and cigarette smoking on OL and OSF was also reported. Compared to individuals who neither chew BQ nor smoke cigarettes, those with both habits had a 40-fold (95% CI = 16.3–99.2) increase in risk of OL and a 57.9-fold (95% CI = 16.0–209.6) increase in risk for OSF.

In addition to case-control studies, three community surveys employed dentists to conduct oral examinations to diagnose OSF and OL in order to investigate the effects of BQ chewing on precancerous oral lesions. Yang et al [19] screened over 2000 participants and found that BQ chewing increased men's risk for OL and OSF, respectively, by 6.57-fold (95% CI = 3.51–12.28) and 22.86-fold (95% CI = 7.28–71.73), and increased women's risk for OL and OSF, respectively, by 15.63 fold (95% CI = 8.31–29.39) and 13.03-fold (95% CI = 5.21–32.62). The results also showed a significant positive association between chewing frequency and risks of OL and OSF, as well as between years of chewing and risks of OL and OSF. That is, heavier chewers with longer durations had higher risks of OL and OSF than lighter chewers with shorter durations of chewing.

A second large-scale study surveyed 1075 adults in southern Taiwan. It found that quid chewers' risk of oral precancerous lesions (OL, OSF, and verrucous lesions) were 8.4 times (95% CI = 5.13–13.75) higher than nonchewers [20]. This study also found a significant synergistic effect of BQ chewing, cigarette smoking, and alcohol drinking on OL. Compared to individuals who did not use BQ, cigarettes, or alcohol, those with reported regular use of all three substances had a 15.12-fold (95% CI = 6.34–36.05) increase in risk of OL.

Given the high prevalence of BQ chewing among aborigines, their risks of oral lesions were specifically examined in a study that screened 312 individuals from an aboriginal community in southern Taiwan [12]. Results showed that the odds of developing either OSF or OL were 8.21-fold (95% CI = 1.80–37.46) higher among chewers than among nonchewers.

So far, five studies have found significant results confirming the etiological role of BQ chewing in the development of oral precancerous conditions. These oral lesions have often been found to be precursors of oral cancer [15,16]. Two case-control studies that examined the effect of BQ chewing on the malignant transformation from oral lesions to oral cancer yielded mixed results. In one study that examined a cohort of 435 individuals diagnosed with OL, BQ chewers were 4.59 times (95% CI = 1.25–16.85) more likely than nonchewers to experience a malignant transformation to oral cancer [21]. However, another study that examined 104 histologically confirmed OSF patients failed to find a significant effect of BQ chewing on the development of oral cancer among OSF patients [22].

3.2. *Oral, esophageal, and pharyngeal cancer*

Four case-control studies investigated the association between BQ chewing and oral, esophageal, and pharyngeal cancer. In a study that enrolled 107 cancer patients and 200 matched noncarcinoma patients, researchers found that chewers had a significantly higher risk than nonchewers of developing oral cancer (OR = 6.9, 95% CI = 3.1–15.2) [23]. Among BQ chewers, those who also smoked cigarettes saw their risk of oral cancer increased from 6.9 times to 89 times (95% CI = 10.0–790.7) higher than those who did not use either BQ or cigarettes.

Two studies reported a positive association between BQ chewing and squamous-cell carcinoma of the esophagus. Both case-control investigations paired patients with histologically confirmed esophageal cancer with hospital-matched noncancer (control) patients. Wu et al [24] recruited 104 cases and 277 controls and found a significant dose-response effect of BQ chewing on esophageal cancer. Compared to nonchewers, patients who consumed ≤495 betel-years (about 20 quids/day for 20 years) had a 3.6-fold (95% CI = 1.3–10.1) increase in risk of esophageal cancer; those who consumed >495 betel-years had a 9.2-fold (95% CI = 1.8–46.7) increase in risk of esophageal cancer.

Lee et al [25] found, in a multi-site case-control study that included 513 esophageal cancer and 818 hospital-matched control patients, that BQ chewers had a 2.3-fold (95% CI = 1.4–3.7) increase in esophageal cancer than non-chewers. They also found a significant association between risk of esophageal cancer and initiation age of BQ chewing, years of chewing, and average amount of quid consumed/day. Furthermore, a synergistic effect of BQ chewing and cigarette smoking was found. Compared to individuals who neither smoked nor chewed, adding cigarette smoking to BQ chewing increased patients' risk of esophageal cancer from 2.3-fold to 8.8-fold (95% CI = 5.2–14.8).

In a study that examined 148 patients with histologically confirmed pharyngeal cancer, 128 patients with histologically confirmed laryngeal cancer, and 255 matched control patients, the results showed that BQ chewing was significantly associated with pharyngeal (OR = 6.9, 95% CI = 3.4–14.3) but not laryngeal cancer [26]. Compared to nonchewers, patients who chewed more than 20 quids/day (OR = 7.2, 95% CI = 3.6–14.8) were also found to incur a significantly higher risk of pharyngeal cancer than those who chewed less than 20 quids/day (OR = 2.5, 95% CI = 1.0–3.8). Adding cigarette smoking to BQ chewing significantly increased patients' risk of pharyngeal cancer from 6.9-fold to 19.0-fold (95% CI = 5.7–70.6). Chewers who swallowed the BQ juice had higher odds (OR = 8.7) of developing pharyngeal cancer than nonswallowers (OR = 6.2). The pharynx, which is located immediately posterior to the mouth, may be more likely than the larynx, which is located in the upper air way and is inferior to the pharynx, to come into direct contact with BQ. As such, the differential anatomical position of the two structures may explain why BQ chewing is significantly associated with pharyngeal but not laryngeal cancer.

In addition to the case-control studies, two cohort studies also found evidence that supported a significant association between BQ chewing and oral cancer. The first study screened 8356 male patients (191 patients were diagnosed with oral cancer) over 2 years at a Taichung hospital and found a significant effect of BQ chewing on the development of oral cancer (OR = 9.03, 95% CI = 3.22–37.34) [27]. The OR of oral cancer increased to 21.79 (95% CI = 11.08–42.85) for patients who chewed BQ and smoked cigarettes.

Finally, information from 177,271 men who participated in a medical screening program was included in a large cohort study that examined the relationship between BQ chewing and cancer mortality [28]. In this study, the majority (90%) of those who chewed BQ also reported cigarette smoking. Compared to men who did not chew BQ or smoke cigarettes, BQ chewers were found to have a significantly higher risk of oral cancer

death [hazard ratio (HR) = 12.52, 95% CI = 5.45–28.77]. BQ chewers were also found to have elevated risk for esophageal cancer death (HR = 5.64, 95% CI = 2.25–14.12), liver cancer death (HR = 2.27, 95% CI = 1.12–4.60), pancreatic cancer death (HR = 2.67, 95% CI = 1.23–5.78), larynx cancer death (HR = 6.24, 95% CI = 1.03–37.44), and lung cancer death (HR = 2.43, 95% CI = 1.73–3.41). Since 90% of the BQ chewers also smoked cigarettes, efforts were made to tease apart the unique contribution of BQ chewing to cancer mortality. Compared to smokers who did not chew BQ, smokers who chewed had a significantly higher hazard of oral cancer death (HR = 4.84, 95% CI = 2.68–8.72), esophageal cancer death (HR = 2.20, 95% CI = 1.12–4.02), liver cancer death (HR = 1.73, 95% CI = 1.35–2.23), and larynx cancer death (HR = 4.09, 95% CI = 1.33–12.55).

In summary, findings from case-control, survey, and cohort studies have revealed a significant effect of BQ chewing on the development of precancerous oral lesions, oral cancer, esophageal cancer, and pharyngeal cancer. These risks were found to be considerably compounded if BQ chewers also smoked cigarettes. Both chewing BQ and smoking have been found to be associated with oral, esophageal, liver, pancreatic, larynx, and lung cancer mortality.

4. Behavioral research program on BQ chewing

Although extensive research findings have linked BQ chewing with oral cancer and precancerous conditions, to date, no pharmacological or behavioral treatments exist for BQ cessation. Previous studies that examined Taiwanese BQ chewers have found that male gender [3–6], membership in an aboriginal tribe [5,9,11], low socio-economic status [3,9], cigarette smoking [13], and alcohol drinking [9,10] are significantly associated with BQ use [3]. While these findings provide valuable information about who uses BQ (i.e., the characteristics of BQ users) in Taiwan, they offer relatively few insights about why these users chew BQ. Better understanding of the reasons why chewers consume BQ is likely to aid researchers in developing treatment programs.

In tobacco research, findings from behavioral studies have contributed significantly to the understanding of nicotine addiction as well as to the development of smoking cessation interventions. For example, several behavioral models of drug addiction have been proposed, which have enriched our understanding regarding smokers' motivation to smoke [29,30] as well as the importance of smoking-related cues in eliciting smoking behaviors [31,32]. Behavioral studies have also uncovered a positive association between nicotine withdrawal symptoms and cessation failure [33–36]. Furthermore, behavioral researchers have found evidence that many behavioral and cognitive constructs (e.g., smoking outcome expectancies, self-efficacy) predict smoking relapse [37,38]. An enormous trove of studies exists on each of these topics and a comprehensive review is beyond the scope of the present article. Thus, we briefly review several areas of behavioral research on smoking and discuss their potential application to BQ chewing.

Considering the contributions that behavioral research has added to the understanding of smoking and smoking cessation, we propose that similar efforts should be initiated to define the behavioral aspects of BQ chewing. To better understand the different facets of BQ chewing, we suggest that behavioral researchers: (1) examine the factors relating BQ chewing initiation; (2) develop a self-report instrument that measures BQ dependence; (3) investigate chewers' motivation to chew BQ; and (4) document withdrawal symptoms experienced by chewers upon stopping BQ.

4.1. *Examination of factors for BQ chewing initiation*

Earlier smoking research focused on individual differences in sensitivity to nicotine, personality, and psychopathology to explain differences in smoking initiation. For example, individuals who showed high sensitivity to their initial exposure to nicotine [39], those with neurotic personality [40], those who suffered from major depression or schizophrenia [41], and those who met criteria for conduct or oppositional defiant disorder [42–44] were at higher risk of becoming smokers. Other studies showed that differences in life experiences such as poor or abusive childhood [45,46], major negative life events [47], and acute or chronic life stressors [48,49] increased a person's risk of taking up smoking. Furthermore, energy-balance factors, such as weight control, have been found to influence adolescents, especially young women, in starting smoking [50,51].

While there are many cross-sectional studies that reported demographic differences between BQ chewers and non-chewers [3,5,10], relatively few studies (cross-sectional or longitudinal) have looked at the role that innate constitutional (e.g., sensitivity to arecoline), psychological, or life experiential factors play in BQ initiation. Understanding not only who but also how a person takes up BQ chewing, and how one progresses from experimental to dependent chewers is likely to prove useful in the future development of BQ cessation programs.

4.2. *A self-report instrument to measure BQ dependence*

Previous studies have shown that nicotine dependence is a significant inverse predictor of success in long-term smoking cessation [52–54]. That is, smokers with a high level of nicotine dependence are significantly less likely than those with a low level of dependence to achieve and maintain smoking abstinence. Furthermore, nicotine dependence has also been found to be associated with psychiatric and other drug dependence disorders [41,55–57].

Nicotine dependence is typically measured using the Fagerström Test for Nicotine Dependence [58], a six-item self-report instrument that assesses various components of smoking behavior such as daily intake, difficulty in refraining from smoking, and time to first cigarette of the day. The items were derived based on smokers' dose (e.g., number of cigarettes smoked/day) and on behaviors often observed in dependent smokers (e.g., earlier smoking in the morning, difficulty refraining from smoking in places where smoking is prohibited). The Fagerström Test for Nicotine Dependence has been shown to be reliable and smokers' scores on the measure are significantly correlated with biochemical markers of nicotine use [58].

BQ chewing is an addictive behavior and chewers are likely to differ in their dependence on the BQ. A self-report instrument to assess BQ dependence will provide researchers with an easy to administer and valid mean to quantify chewers' dependence. With such an instrument, researchers can begin to examine questions regarding dependence (e.g., are all BQ chewers dependent?), consumption (e.g., do more dependent chewers chew more BQs/day?), and abstinence (e.g., do more dependent chewers have more difficulty quitting than their less dependent counterparts?). A self-report dependence scale will also allow researchers to examine the role that active quid ingredients (e.g., arecoline, arecaidine) plays in BQ use, and the complex ways in which these ingredients interact with behavioral and sensory factors in determining BQ consumption.

Two studies have shown that arecoline can be reliably detected, using high-performance liquid chromatography, in BQ chewers' saliva [59] and blood [60]. Wu et al [60] further demonstrated that the levels of plasma arecoline and plasma arecaidine found in BQ chewers' blood were positively correlated with the amount of BQ consumed, making them ideal biochemical markers of BQ use. Future behavioral research should consider employing plasma arecoline or arecaidine levels to validate self-report dependence measure or to verify self-report BQ use and cessation.

4.3. *Chewers' motivation to chew BQ*

Smoking behaviors are often influenced by smoking outcome expectancies. It has been hypothesized that while smokers with high expectancies for positive outcome of smoking (e.g., positive mood enhancement, alleviation of negative mood) are likely to increase their cigarette consumption, those with high expectancies for negative smoking outcome (e.g., smoking is detrimental to one's health) are likely to reduce their cigarette intake. Several studies have found that positive smoking outcome expectancies significantly predict the occurrence of smoking lapse and relapse [61–63].

Many BQ chewers expect that chewing can improve their mood, heighten their alertness, quench their thirst, warm their body, curb their appetite, and increase their stamina [64]. They also know that chewing increases their risk of oral cancer [3]. Nevertheless, it is unclear whether or not these positive and negative outcome expectancies of chewing influence chewers' behaviors. To better understand chewers' motivation to use or to quit BQ, it is imperative for behavioral researchers to begin to examine these factors and their effects on chewing.

4.4. *BQ withdrawal symptoms*

When smokers stop smoking, they experience nicotine withdrawal symptoms that may include a negative affect (e.g., depression), urge to smoke, irritability, anxiety, cognitive and attention deficits, sleep disturbance, and increased appetite. The nature of postcessation withdrawal symptoms may have important implications for smoking relapse. If postcessation withdrawal symptoms resolve quickly, abstinent smokers may be less motivated to smoke and may have better success in maintaining their abstinence. However, if postcessation withdrawal symptoms are unrelenting, abstinent smokers may be more driven to smoke and may eventually resort to smoking for withdrawal relief. Numerous studies have found evidence that supports a strong link between postcessation withdrawal symptoms and smoking lapse or relapse. For example, Piasecki et al [33–36] identified several different trajectories of nicotine withdrawal and found that abstinent smokers who had prototypical withdrawal profile (transient course with quick resolution of symptoms) were significantly less likely to lapse than those who had the atypical profiles (extended course of unrelieved symptoms).

Specific nicotine withdrawal symptoms have also been found to be directly and indirectly associated with smoking and smoking relapse. At least one model of addiction has examined the effects of negative affect on smoking [29] and postcessation negative affect has been found to be one of the most potent predictor of relapse [65–69]. In addition to negative affect, smoking urge has also received a lot of attention in smoking cessation research. Several studies have found a positive link between urge and smoking relapse [70,71]. Finally, smokers often use smoking as a mean to control their body weight. Several studies have found evidence suggesting that smokers, especially women, who quit smoking resumed their cigarette consumption to curb increased appetite, a symptom of nicotine withdrawal [72–75].

Given this evidence regarding the effect of withdrawal symptoms on smoking relapse, we argue that it is important to study BQ withdrawal symptoms and to track changes in them across the postcessation period. Anecdotal evidence suggests that BQ chewers may experience withdrawal symptoms when they stop chewing. However, to date, no systematic efforts have been made to investigate what constitutes BQ withdrawal. Thus, we suggest that behavioral researchers to begin to identify the nature and frequency of all symptoms of withdrawal that BQ chewers experience when they abstain from chewing, and to track these overtime.

5. Summary

As many as 10% of people in Taiwan chew BQ. A typical chewer is male, middle-aged, living in a rural area, has a low level of education, and works in a blue-collar occupation. Among chewers, those who do not use alcohol and have a higher level of education are more successful in quitting. Despite a strong link between BQ chewing and oral cancer, relatively few efforts have been made to examine why chewers use BQ and the behavioral sequelae that are related to BQ addiction. We suggest several areas (dependence, drug use motivation, and withdrawal symptoms) in which researchers can begin to study the behavioral aspects of BQ chewing.

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Review article

Very long non-coding RNA and human disease

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ABSTRACT

A role for non-coding RNAs (ncRNAs) in the development of disease has been well documented in the case of miRNAs. Recent studies have shown that long non-coding RNAs (lncRNAs), greater than 200 nt in length, are also implicated in various diseases. In this review, we focus on these lncRNAs, the very long non-coding RNAs (vlncRNAs), which are more than 5 kb long and for which detailed information is available. These studies have demonstrated that vlncRNAs have important biological functions, and that their aberrant expression may result in various cancers. Future investigations in this exciting field are needed to explore the role of vlncRNAs in pathogenesis and, in particular, to further understand their functional mechanisms.

1. Introduction

Classically, proteins are recognized as having the main responsibility for biological function, with RNA merely a messenger that transfers protein-coding information from DNA [1,2]. This concept has changed in recent years, however—whereas only 2% of the genome encodes protein, more than 80% of the genome produces non-protein coding RNA transcripts [1–5], and these non-coding RNAs (ncRNAs) have important biological functions including gene regulation [6,7], imprinting [8–12], epigenetic regulation [13,14], cell cycle control [15], regulation of transcription, translation, and splicing [7,16–20].

There are two major classes of ncRNAs, grouped according to size: small RNA, which includes microRNA (miRNA), PIWI-interacting RNA (piRNA), endogenous short interfering RNA (endo-siRNA), and other ncRNAs less than 200 nt; and long non-coding RNA (lncRNA), which is larger than 200 nt and is transcribed from intergenic, intragenic, or around protein-coding regions. miRNAs are involved in post-transcriptional regulation of mRNA through the RNA-induced silencing complex [16],

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whereas piRNAs and siRNAs maintain genomic integrity by suppressing transposable elements [17] or other unknown factors in cell nucleus [18]. The lncRNAs are involved in various levels of genome regulation and related fundamental epigenetic processes [19–25].

The importance of lncRNAs in gene regulation has become apparent in recent years [6,20–30], but the key sequences of lncRNA that determine regulatory function remain unknown. Thus, although the rules of translation via the genetic code are well understood and a mutation in a protein-coding gene that contributes to a given disease can be attributed to the resultant change in amino acid sequence [6,20–30], there is no equivalent code for lncRNA function. Genetic studies on lncRNA could help us identify their regulatory sequences and understand their mechanism of action more clearly. In this review, we focus on a specific group of lncRNAs, those more than 5000 nt long, which we call very long non-coding RNAs (vlncRNAs), and explore their roles in the development of disease.

2. vlncRNA annotation and relevant databases

High-throughput technologies such as Tiling Chip or Deep Sequencing data, combined with computational approaches, have identified long, abundantly expressed non-coding transcripts associated with various cancers [13,28,29,31–37]. Currently, ncRNAs are curated by a variety of public databases, such as lncRNAdb (http://lncrnadb.com/), a database of eukaryotic lncRNAs validated by experimental data [38]; RNAdb (http://research.imb.uq.edu.au/rnadb/), a lncRNA set conserved between human and mouse that was used in a high-throughput functional screen [39]; fRNAdb (http://www.ncrna.org/frnadb/index.html), a database hosting a large collection of ncRNA sequence data from public non-coding databases [40]; NONCODE (http://www.noncode.org/NONCODERv3/), a database of non-coding RNAs [41], together with annotation of potential function, based on a coding–non-coding coexpression network [34]; and NRED (http://jsm-research.imb.uq.edu.au/nred/), a database of expression data of human and mouse lncRNAs with various gene expression profiles [31]. Moreover, the Encyclopedia of DNA Elements consortium is annotating lncRNAs using a combination of RNA-seq data, chromatin state maps, and computational approaches [42]. In this review, we survey the vlncRNAs, collected from various ncRNA databases by filtering for sequence length greater than 5000 nt.

3. Function of vlncRNAs

vlncRNAs originate from intronic, exonic, intergenic, intragenic, and promoter regions, and from 3′- and 5′-UTRs and enhancer sequences; they are sometimes bidirectional transcripts [30]. In particular, a large group of vlncRNAs, referred to as natural antisense transcripts, is antisense to known protein-coding genes [43,44]. In the following sections, we will discuss various vlncRNAs with respect to their functions in epigenetic regulation, transcriptional and post-transcriptional regulation, as well as in tumorigenesis (Fig. 1).

4. vlncRNAs control transcription by mediating changes in chromatin structure

The structure of chromatin determines the accessibility of DNA to polymerase II and transcription factors, and is integral to transcriptional control. Chromatin structure can be altered by specific post-translational modification, such as trimethylation of histone H3 lysine 4 (H3K4me3) at gene promoters, whereas H3K36me3 in transcribed regions is linked to gene activation, and H3K9me3, H3K27me3, and H4K20me3 are linked to repression [45] (Fig. 1A–C). There is substantial evidence for an important role of lncRNAs in these processes, and

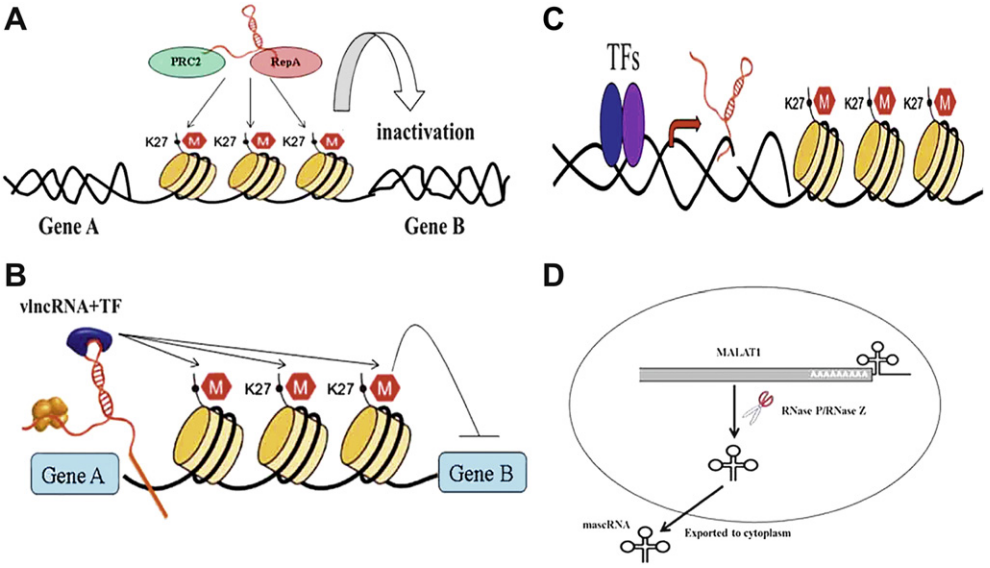

Fig. 1 – Mechanisms for regulation of epigenetics and gene expression by vlncRNAs. (A) vlncRNA regulates histone modification in *cis* and in *trans*. (B) Promoter-associated vlncRNA as an inhibitor of transcription. (C) Promoter-associated vlncRNA as an activator of transcription. (D) vlncRNA generation of sRNA regulatory transcript.

approximately one-third of long intergenic non-coding RNA (lincRNA) is associated with chromatin-modifying complexes [13]. In addition, many intronic and intergenic ncRNAs have been found by different methods [14], suggesting that lncRNAs are key participants in controlling the chromatin structure. Similar to lncRNAs, vlncRNAs are also associated with chromatin-modifying complexes and can affect gene expression. The following examples describe vlncRNAs that mediate changes in chromatin structure and control transcription.

4.1. *XIST control of X-chromosome inactivation*

On X-chromosome inactivation (XCI), one of the two X chromosomes in female mammals is inactivated to achieve comparable expression levels of X-chromosome genes in males and females. A number of vlncRNAs including *XIST* and *TSIX* participate in this process [46–49]. There are data suggesting that *XIST* recruits chromatin modeling complexes to silence the X chromosome (resulting in an inactive X chromosome, denoted Xi). Zhao et al [50] discovered a 1.6-kb ncRNA (*RepA*) transcribed from the 5′ region of *XIST*, which binds polycomb repressive complex 2 (PRC2) to bring about silencing (Fig. 1A). In pre-XCI cells, *RepA* initially recruits PRC2 to the future Xi and inhibits the interaction of *TSIX* by binding PRC2. During initiation of the silencing process, *TSIX* is down-regulated on the future Xi, and *RepA* can engage PRC2 resulting in the activation of full-length *XIST* transcription (Fig. 1B and C). The up-regulated *XIST*, in turn, preferentially binds to PRC2 through its *RepA* sequence, resulting in the spread of *XIST* along Xi and the distribution of PRC2 and trimethylated histone H3K27 throughout the Xi [46,47,50]. Therefore, *RepA* and *XIST* are capable of recruiting PRC2 to establish the local chromatin modification required for the initiation and spread of XCI, resulting in the suppression of expression of genes on the Xi.

4.2. *vlncRNAs involved in imprinting*

Just as vlncRNAs play important roles in X-inactivation, similar mechanisms have also been observed during genomic imprinting [8–10]. *AIR*, a 108-kb-long vlncRNA, is required for allele-specific silencing of the *cis*-linked *SLC22A3*, *SLC22A2*, and *IGF2R* genes [11]. *AIR* interacts with the *SLC22A3* promoter chromatin and the H3K9-specific histone methyltransferase G9a in placenta [10]. Depletion of G9a fails to silence *SLC22A3* and results in nonimprinted transcription. When truncated, *AIR* does not accumulate at the *SLC22A3* promoter, resulting in reduced G9a recruitment and biallelic transcription [10]. Similarly, the 90.5-kb vlncRNA *KCNQ1OT1* has been linked to the bidirectional silencing of about 10 paternally imprinted genes in the *KCNQ1OT1* domain, and the mechanism involves the interaction between *KCNQ1OT1* and both G9a and PRC2 in a lineage-specific manner [12].

4.3. *vlncRNAs involved in epigenetic silencing*

The *INK4b/ARF/INK4a* locus in human cells contains three important tumor suppressors, whose expression is controlled in part by PRC1, PRC2, and histone methylation [51]. A vlncRNA called *ANRIL* (antisense non-coding RNA in the INK4 locus) is transcribed in an antisense direction to the protein-coding genes in this locus, and is up-regulated in some cancer tissues [52]. *ANRIL* plays an important role in controlling the epigenetic state of the *INK4b/ARF/INK4a* locus through interactions with subunits of PRC1 and PRC2. In brief, *ANRIL* binds to and recruits PRC1 and PRC2, resulting in trimethylation of H3K27 and repression of the *INK4b/ARF/INK4a* locus, facilitating oncogenesis [52] (Fig. 1A).

5. vlncRNA regulates splicing

The vlncRNA *MALAT1* (metastasis-associated in lung adenocarcinoma transcript 1) was identified in an attempt to characterize transcripts associated with early-stage non-small cell lung cancer (NSCLC) [53]. Two recent studies found that *MALAT1* regulates alternative splicing through its interaction with the serine/arginine-rich (SR) family of nuclear phosphoproteins involved in the splicing machinery [7,54], and *MALAT1* has been suggested to serve as a fine-tuning mechanism to modulate the activity of SR proteins.

MALAT1 is an abundant vlncRNA transcribed from chromosome 11q13 and primarily localized in nuclear speckles. It modulates the distribution of pre-mRNA splicing factors to nuclear speckles and particularly affects the phosphorylation state of SR proteins [54]. In *MALAT1*-depleted cells, levels of mislocalized and unphosphorylated SR proteins increase, resulting in a higher number of exon inclusion events [7]. Therefore, *MALAT1* contributes to a broad post-transcriptional gene-regulatory mechanism by coordinating a specific mRNA patterning in distinct cell types.

6. vlncRNA is involved in a wide variety of other biological processes

Apart from their roles in nuclear processes discussed above, vlncRNAs have also been implicated in the regulation of a number other biological processes. For example, some vlncRNAs can act as precursors for small RNAs, either by an RNase III-like cleavage from the sense and antisense duplexes, such as *XIST/TSIX*, or by a tRNA-like 3′-end processing of *MALAT1* [43,44] (Fig. 1D). Another example is the vlncRNA *DLEU2* (deleted in lymphocytic leukemia 2), which is frequently deleted in malignancy and functions as a critical host gene of the cell cycle inhibitory miR-15/16 [55]. Finally, *P15AS* (P15-antisense), which overlaps a protein-coding gene but is transcribed in the opposite direction, facilitates cancer progression by silencing its parental gene in *cis* and in *trans* through the formation of a type of heterochromatin [52,56].

7. vlncRNAs and diseases

The data gathered to date strongly implicate vlncRNAs in the basal regulation of protein-coding genes, including those central to normal development and oncogenesis, at both the transcriptional and post-transcriptional levels, and an increasing number have been functionally validated as affecting different cellular and developmental pathways [57,58]. It is not surprising, therefore, that the dysregulation of vlncRNAs appears to be a primary feature of many complex human diseases, including cancer.

Here, we describe some of the better characterized vlncRNAs that have been associated with cancer biology (Table 1).

7.1. Involvement in various cancers

XIST expression levels are correlated with outcome in some cancers [59,60], such as the therapeutic response in ovarian cancer [61], and it is frequently implicated in breast cancer [62–64]. Notably, however, because *XIST* is not expressed in males, such correlations are only of value in samples obtained from females [65].

7.2. Involvement in metastasis

The *MALAT1* gene was associated with high metastatic potential and poor patient prognosis during a comparative screen of NSCLC patients with and without metastatic tumors [53]. Another report indicated that up-regulated *MALAT1* contributes to bladder cancer cell migration by inducing epithelial–mesenchymal transition-associated genes [66].

7.3. Cancer type-specific vlncRNA expression

Many studies describe vlncRNA expression in various cancers, but a few vlncRNAs are specifically expressed in a particular type of cancer. For example, the vlncRNAs *PCA3* (prostate cancer antigen 3), *PCGEM1* (prostate-specific transcript 1), and *PCNCR1* (prostate cancer non-coding RNA 1) are associated with prostate cancer [67–70]. Indeed, *PCA3* is a very sensitive and specific molecular marker for the diagnosis of prostate cancer [71]. Likewise, expression of *PCGEM1*, a prostate-specific gene with a function in the regulation of apoptosis, is associated with high-risk prostate cancer patients [70]. The vlncRNA *PCNCR1* is also involved in prostate cancer progression [68].

8. Perspectives

As in the case of lncRNAs, vlncRNAs do not have protein-coding capacity, but nevertheless have functions relating to the programming and regulation of the mammalian genome. There have been recent rapid advancements in the understanding of these functions, but several important problems remain to be solved. For example, vlncRNAs frequently exhibit sequence divergence but have conserved functions, perhaps indicating the importance of secondary structure. Moreover, although a number of vlncRNAs are associated with PRC2 complexes, they do not interact exclusively with these proteins. Therefore, it will

Table 1 – Identification of tumor-associated vlncRNAs.

vlncRNA	Molecular mechanism	Tumor	Reference
P15AS/ANRIL	Antisense, transcription regulation	Prostate cancer	[52,56]
BA318C17.1	Unknown	Colon cancer	[72]
GNAS-AS1	Unknown		[73]
His-1 RNA	Unknown		[74]
KCNQ1OT1	DNMT1 interaction and transcription gene silencing	Colon cancer	[8,12]
DLEU2	Primary miRNA, other	Chronic lymphocytic leukemia	[55,75]
LOC285194	Unknown	Osteosarcoma	[76]
MALAT1	RNA splicing, small RNA Production, protein interaction	Multiple cancers	[44,53,66,77]
MEG3	Unknown	Multiple cancers	[78,79]
NCRMS	Unknown		[80]
NTT sense/antisense	Unknown		[81,82]
p53 mRNA	RNA protein binding	Multiple cancers	[83]
p53int1	unknown		[84]
PCA3/DD3	Unknown	Prostate cancer	[67]
PCGEM1	Unknown	Prostate cancer	[70]
PCNCR1	Unknown	Prostate cancer	[68]
PR Antisense	Regulation of gene expression		[85]
PRINS	Unknown		[86,87]
SRA RNA	RNA–protein binding, transcription factor coactivator	Breast cancer	[88,89]
TSIX	Antisense of Xist		[62,90]
SNHG14/UBE3A-AS1	Unknown		[91,92]
UCA1	Unknown	Bladder cancer	[93,94]
XIST	X inactivation	Multiple cancers	[59,60,62,95]
ZNFX1-AS1	Unknown	Breast cancer	[96]

be of great interest to unravel the sequences and structural motifs in vlncRNAs that determine their function.

Another challenging unanswered question is how protein partners interact with vlncRNAs to bring about their specialized functions. vlncRNAs, similar to lncRNAs, may recruit and then guide their protein partners to the correct chromosomal destinations. Specific sequences within vlncRNAs could recognize particular chromatin regions via sequence complementarity, thereby bringing the associated proteins to the targeted region. For example, *XIST* recruits PRC2 to establish local chromatin modification on Xi [50]. In the case of vlncRNAs recruiting proteins at a distance [34,35] or in *trans*, the higher-order, tertiary structure of chromatin might help bring distant chromosomal regions together. Alternatively, vlncRNAs might induce allosteric structural modifications of their protein partners to either enhance or suppress their normal activities.

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Clinical spotlight

Unusual rectal submucosal tumor in melanosis coli

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A 60-year-old female patient was referred for polypectomy of multiple colonic flat adenomatous polyps. She suffered no abdominal pain or decreased stool caliber, and tested negative for stool occult blood. Her medical history revealed that she had been taking a laxative for 20 years as a treatment for constipation. The patient denied any systemic disease or taking any medication except for the laxative. Colonoscopy revealed multiple whitish flat polyps in melanosis coli throughout the entire colon, the largest of which was approximately 1.5 cm over the ascending colon (Fig. 1). A submucosal nodule, approximately 5 mm in size (Fig. 2), with multiple black dots on its surface was revealed when the scope was withdrawn to the rectum. Endoscopic ultrasound revealed a tumor originating in the second layer of the rectal wall, which was approximately 5 mm in size (Fig. 3).

A polypectomy of the rectal submucosal tumor was performed (Fig. 4). The mucosa was abnormally dark brown to black, grossly consistent with the presence of melanosis coli. Histopathologic evaluation with hematoxylin and eosin (H&E) staining (Fig. 5) showed that pigment-laden macrophages consistent with melanosis coli were present in the lamina propria and that tumor cells were arranged in trabecular and glandular patterns, characterized by scanty cytoplasm, round nuclei, and fine chromatin, with rare mitotic figures. Immunohistochemical staining results were positive for markers of synaptophysin,chromogranin A (Fig. 6), and neuron-specific enolase. Rectal carcinoid in melanosis coli was identified.

The overall incidence of carcinoid tumors is difficult to determine because many are asymptomatic. They are discovered incidentally during routine colonoscopy and are usually less than 13 mm in size. Small rectal carcinoids are rarely malignant, and endoscopic resection is curative. The prevalence of rectal carcinoid is on the rise, particularly in the United States, where its age-adjusted incidence has increased by 800–1000% in the last 35 years [1]. The prevalence of rectal carcinoid in adults, as revealed by colonoscopy, is 0.05–0.07% [2].

Melanosis coli refers to an abnormal brown or black pigmentation of colonic mucosa caused by the presence of lipofuscin produced by the breakdown of apoptotic colonic epithelial cells in macrophages within the lamina propria. Melanosis coli is usually related to chronic use of laxative agents. Endoscopic observation reveals blackening over the

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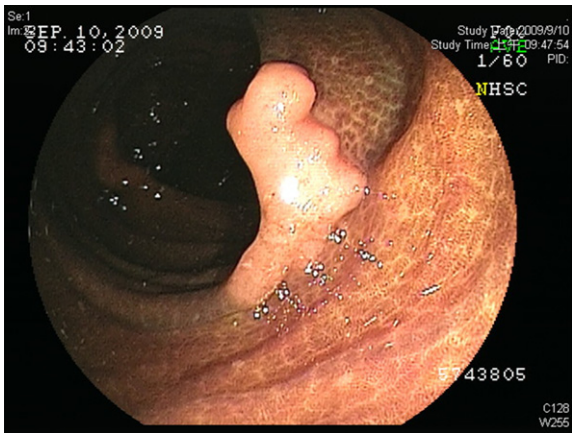

Fig. 1 – Colonoscopic image showing whitish flat polyps in melanosis coli of ascending colon.

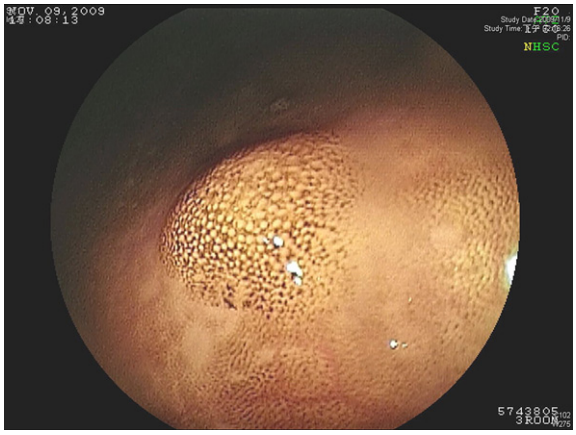

Fig. 2 – Colonoscopic image revealing a submucosal nodule with multiple black dots in rectum.

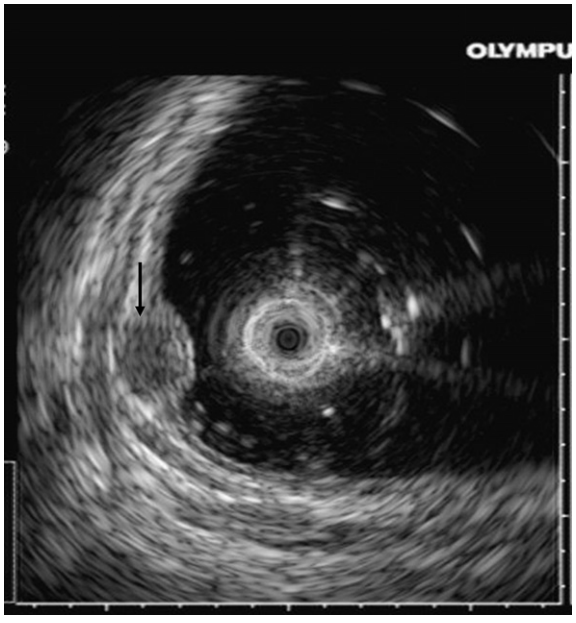

Fig. 3 – Endoscopic ultrasound shows a tumor, approximately 5 mm in size, originating in the second layer of rectal wall.

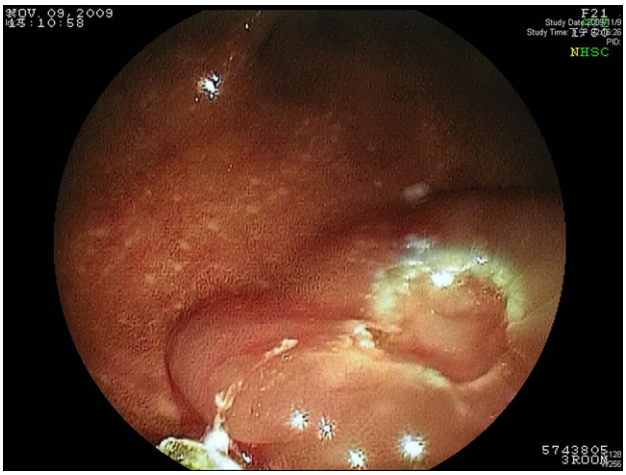

Fig. 4 – Postpolypectomy of rectal submucosal tumor.

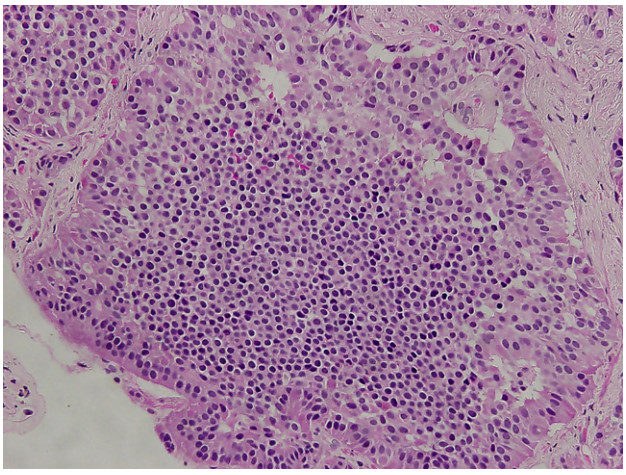

Fig. 5 – H&E staining shows that melanosis coli is present in the lamina propria, with tumor cells arranged in trabecular and glandular patterns characterized by scanty cytoplasm, round nuclei, and fine chromatin.
H&E = hematoxylin and eosin.

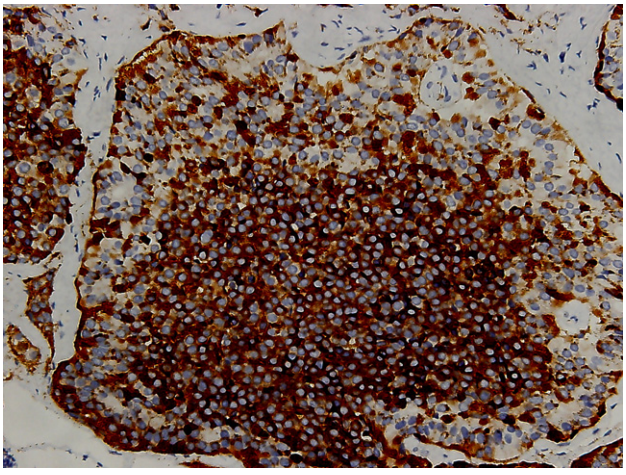

Fig. 6 – Synaptophysin staining shows positive findings.

entire colonic mucosa [3]. Epithelial neoplasms in melanosis coli are easily observed because of their whitish color against a black background, as in the present case.

Rectal carcinoid usually presents a yellowish color change in the submucosal tumor, but this color change is often covered by black colonic mucosa. Submucosal tumor will be distal to mucosa, revealing multiple black dots in a diffuse black background under the endoscopic observation, as in this case. In conclusion, caution must be exercised in detecting submucosal tumor in melanosis coli.

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The Editorial Board requires authors to be in compliance with the *Uniform Requirements for Manuscripts Submitted to Biomedical Journals* (URMs); current URMs are available at http://www.icmje.org.

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Manuscripts should be submitted online through Elsevier's Editorial System (EES). This system can be accessed at http://ees.elsevier.com/biomed. This site will guide authors stepwise through the submission process. If assistance is required, please refer to the tutorials and/or customer support that are available on the website, or you may contact the Editorial Office.

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1.1. *Important Information*

- Articles submitted should be in Microsoft Word document format and prepared in the simplest form possible. We will add in the correct font, font size, margins and so on according to the journal's style.
- You may use automatic page numbering, but do NOT use other kinds of automatic formatting such as footnotes, headers and footers.
- Put text, references, and table/figure legends in one file.
- Figures must be submitted separately as picture files, at the correct resolution. The files should be named according to the figure number, e.g., "Article1_Fig1", "Article1_Fig2". Also see Section 9.7. below.

1.2. *Supporting Documents*

The following documents must be included (refer also to the Checklist that follows these author instructions):

(1) Cover Letter. This must include the name, address, telephone and fax numbers, and e-mail address of the corresponding author.

(2) Authorship Statement. You may use the form that follows these author instructions. ALL the authors' signatures must be included.

(3) Conflict of Interest Statement. You may use the form that follows these author instructions. Also see Section 2 below.

(4) Copyright Transfer Agreement. You may use the form that follows these author instructions.

(5) Ethics Statement. Articles covering human or animal experiments must be accompanied by a letter of approval from the relevant review committee or authorities. Also see Section 3 below.

(6) Consolidated Standards of Reporting Trials (CONSORT) flow chart for randomized controlled trials submitted for publication. Also see Section 4 below.

(7) Articles where human subjects can be identified in descriptions, photographs or pedigrees must be accompanied by a signed statement of informed consent to publish (in print and online) the descriptions, photographs and pedigrees from each subject who can be identified. Also see Section 5 below.

(8) Where material has been reproduced from other copyrighted sources, the letter(s) of permission from the copyright holder(s) to use the copyrighted sources must be supplied.

2. Disclosure of Conflicts of Interest

All authors are required to sign and submit a financial disclosure statement at the time of manuscript submission, for example:

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Authors who have no relevant financial interests should provide a statement indicating that they have no financial interests related to the material in the manuscript. Any non-financial conflicts of interest should also be explicitly declared in your own words.

3. Ethical Approval of Studies and Informed Consent

For human or animal experimental investigations, appropriate institutional review board or ethics committee approval is required, and such approval should be stated in the methods section of the manuscript. For those investigators who do not have formal ethics review committees, the principles outlined in the Declaration of Helsinki should be

followed (World Medical Association. *Declaration of Helsinki: ethical principles for medical research involving human subjects*. Available at: http://www.wma.net/en/30publications/10policies/b3/index.html).

For investigations in humans, state explicitly in the methods section of the manuscript that informed consent was obtained from all participating adults and from parents or legal guardians for minors or incapacitated adults, together with the manner in which informed consent was obtained (ex. oral or written).

For work involving experimental animals, the guidelines for their care and use should be in accordance with *European Commission Directive 86/609/EEC for animal experiments* (available at http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm); this should be stated in the methods section of the manuscript.

4. Reporting Clinical Trials

All randomized controlled trials submitted for publication should include a completed Consolidated Standards of Reporting Trials (CONSORT) flow chart (available at http://www.consort-statement.org). This Journal has adopted the proposal from the International Committee of Medical Journal Editors (ICMJE) that require, as a condition of consideration for publication of clinical trials, registration in a public trials registry. Purely observational studies (those in which the assignment of the medical intervention is not at the discretion of the investigator) do not require registration. Further information can be found at http://www.icmje.org.

5. Identification of Patients in Descriptions, Photographs and Pedigrees

A signed statement of informed consent to publish (in print and online) patient descriptions, photographs and pedigrees should be obtained from all subjects (parents or legal guardians for minors) who can be identified (including by the subjects themselves) in such written descriptions, photographs or pedigrees. Such persons should be shown the manuscript before its submission. Omitting data or making data less specific to de-identify patients is acceptable, but changing any such data is not acceptable.

6. Previous Publication or Duplicate Submission

Submitted manuscripts are considered with the understanding that they have not been published previously in print or electronic format (except in abstract or poster form) and are not under consideration in totality or in part by another publication or electronic medium.

7. Basic Criteria

Articles should be written in English (using American English spelling) and meet the following basic criteria: the material is original, the information is important, the writing is clear and concise, the study methods are appropriate, the data are valid, and the conclusions are reasonable and supported by the data.

8. Article Categories

8.1. *Review Articles*

These should aim to provide the reader with a balanced overview of an important and topical subject in the field, and should be systematic and critical assessments of literature and data sources. They should cover aspects of a topic in which scientific consensus exists as well as aspects that remain controversial and are the subject of ongoing scientific research. All articles and data sources reviewed should include information about the specific type of study or analysis, population, intervention, exposure, and tests or outcomes. All articles or data sources should be selected systematically for inclusion in the review and critically evaluated.

By invitation only. The format for review articles will be jointly decided by the Editors and the contributing author. Typical length: no more than 4000 words, 50–100 references.

8.2. *Original Articles*

These may be randomized trials, intervention studies, studies of screening and diagnostic tests, laboratory and animal studies, cohort studies, cost-effectiveness analyses, case-control studies, and surveys with high response rates, which represent new and significant contributions to the field.

Section headings should be: Abstract, Introduction, Methods, Results, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

The Introduction should provide a brief background to the subject of the paper, explain the importance of the study, and state a precise study question or purpose.

The Methods section should describe the study design and methods (including the study setting and dates, patients/participants with inclusion and exclusion criteria, or data sources and how these were selected for the study, patient samples or animal specimens used, explain the laboratory methods followed), and state the statistical procedures employed in the research.

The Results section should comprise the study results presented in a logical sequence, supplemented by tables and/or figures. Take care that the text does not repeat data that are presented in tables and/or figures. Only emphasize and summarize the essential features of any interventions, the main outcome measures, and the main results.

The Discussion section should be used to emphasize the new and important aspects of the study, placing the results in context with published literature, the implications of the findings, and the conclusions that follow from the study results. Typical length: no more than 5500 words, 40–80 references.

8.3. *Case Reports*

These are short discussions of a case or case series with unique features not previously described that make an important teaching point or scientific observation. They may describe novel techniques, novel use of equipment, or new information on diseases of importance. Section headings should be: Abstract, Introduction, Case Report, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

The Introduction should describe the purpose of the report, the significance of the disease and its specificity, and briefly review the relevant literature.

The Case Report should include the general data of the case, medical history, family history, chief complaint, present illness, clinical manifestation, methods of diagnosis and treatment, and outcome.

The Discussion should compare, analyze and discuss the similarities and differences between the reported case and similar previously reported cases. The importance or specificity of the case should be restated when discussing the differential diagnoses. Suggest the prognosis of the disease and possibility of prevention. Typical length: no more than 1500 words, 20–40 references.

8.4. *Short Communications*

These should be concise presentations of clinical or preliminary experimental results. Section headings should be: Abstract, Introduction, Methods, Results, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

Typical length: no more than 1000 words, 20–40 references, with no more than four figures or tables. The Editors reserve the right to decide what constitutes a Short Communication.

8.5. *Letters to the Editor*

Letters are welcome in response to previously published articles, and may also include interesting cases that do not meet the requirement of being truly exceptional, as well as other communications of general interest. Letters should have a title and include appropriate references, and include the corresponding author's mailing and e-mail addresses. Letters are edited, sometimes extensively, to sharpen their focus. They may be sent for peer review at the discretion of the Editors. Letters are selected based on clarity, significance, and space. Typical length: no more than 600 words, 5–10 references; 1 table and/or 1 figure may be included.

8.6. *Editorials*

Editorials are invited articles or comments concerning a specific paper in the Journal or a topical issue in the field. While normally invited, unsolicited editorials may be submitted. Typical length: no more than 1500 words, 15–30 references.

9. Manuscript Preparation

Text should be typed double-spaced on one side of white A4 (297 × 210 mm) paper, with outer margins of 2.5 cm. A manuscript should include a title page, abstract, text, acknowledgments (if any), conflicts of interest statement (if any), references, and figures and tables as appropriate. Each section of the manuscript should begin on a new page. Pages should be numbered consecutively, beginning with the title page.

9.1. *Title Page*

The title page should contain the following information (in order, from the top to bottom of the page):

- category of paper
- article title
- names (spelled out in full)* of all the authors, and the institutions with which they are affiliated; indicate all affiliations with a superscripted lowercase letter after the author's name and in front of the appropriate affiliation
- corresponding author details (name, e-mail, mailing address, telephone and fax numbers)

**The name of each author should be written with the family name last, e.g., Jing-Lin Chang. Authorship is restricted only to direct participants who have contributed significantly to the work.*

9.2. *Abstract and Keywords*

Abstracts should be no more than 300 words in length. Abstracts for Original Articles should be structured, with the section headings: Background/ Introduction, Purpose(s)/Aim(s), Methods, Results, Conclusion. Abstracts for Case Reports are unstructured, but should include the significance and purpose of the case presentation, the diagnostic methods of the case, the key data, and brief comments and suggestions with regard to the case. Abstracts for Review Articles and Short Communications should also be unstructured. No abstract is required for Letters to the Editor and Editorials. For the article categories that require an abstract, 3–5 relevant keywords should also be provided in alphabetical order.

9.3. *Main Text*

The text for Original Articles should be organized into the following sections: Background/ Introduction, Purpose(s)/Aim(s), Methods, Results and Discussion. Sections for Case Reports are: Introduction, Case Report, and Discussion. Each section should begin on a new page.

9.3.1. Abbreviations

Where a term/definition will be continually referred to, it must be written in full when it first appears in the text, followed by the subsequent abbreviation in parentheses. Thereafter, the abbreviation may be used. An abbreviation should not be first defined in any section heading; if an abbreviation has previously been defined in the text, then the abbreviation may be used in a subsequent section heading. Restrict the number of abbreviations to those that are absolutely necessary.

9.3.2. Units

Système International (SI) units must be used, with the exception of blood pressure values which are to be reported in mmHg. Please use the metric system for the expression of length, area, mass, and volume. Temperatures are to be given in degrees Celsius.

9.3.3. Names of drugs, devices and other products

Use the Recommended International Non-proprietary Name for medicinal substances, unless the specific trade name of a drug is directly relevant to the discussion. For devices and other products, the generic term should be used, unless the specific trade name is directly relevant to the discussion. If the trade name is given, then the manufacturer name and the city, state and country location of the manufacturer must be provided the first time it is mentioned in the text, for example, "…SPSS version 11 was used (SPSS Inc., Chicago, IL, USA)."

9.3.4. Statistical requirements

Statistical analysis is essential for all research papers except case reports. Use correct nomenclature of statistical methods (e.g., two sample *t* test, not unpaired *t* test). Descriptive statistics should follow the scales used in data description. Inferential statistics are important for interpreting results and should be described in detail.

All p values should be expressed to 2 digits to the right of the decimal point, unless $p < 0.01$, in which case the p value should be expressed to 3 digits to the right of the decimal point. The smallest p value that should be expressed is $p < 0.001$, since additional zeros do not convey useful information; the largest p value that should be expressed is $p > 0.99$.

9.3.5. Personal communications and unpublished data

These sources cannot be included in the references list but may be described in the text. The author(s) must give the full name and highest academic degree of the person, the date of the communication, and indicate whether it was in oral or written (letter, fax, e-mail) form. A signed statement of permission should be included from each person identified as a source of information in a personal communication or as a source for unpublished data.

9.4. *Acknowledgments and Conflicts of Interest Statement*

General acknowledgments for consultations, statistical analysis, etc., should be listed concisely at the end of the text, including the names of the individuals who were directly involved. Consent should be obtained from those individuals before their names are listed in this section. All financial and material support for the research and work from internal or external agencies, including commercial companies, should be clearly and completely identified. Ensure that any conflicts of interest (financial and/or non-financial) are explicitly declared.

9.5. *Abbreviation list*

A term that appears more than three times in a paper should be abbreviated. Spell out the term on first mention, followed by the abbreviated form in parentheses. Thereafter, please use the abbreviated form. Supply a list of nonstandard abbreviations used in the paper at the end of the main text, in alphabetical order, giving each abbreviation followed by its spelled-out version.

9.6. *References*

9.6.1. In the main text, tables, figure legends

- References should be indicated by numbers in square brackets in line with the text, and numbered consecutively in order of appearance in the text.
- References cited in tables or figure legends should be included in sequence at the point where the table or figure is first mentioned in the main text.
- Do not cite uncompleted work or work that has not yet been accepted for publication (i.e., "unpublished observation", "personal communication") as references. Also see Section 9.3.5. above.
- Do not cite abstracts unless they are the only available reference to an important concept.

9.6.2. In the references section

- References should be limited to those cited in the text and listed in numerical order, NOT alphabetical order.
- References should include, in order, author surnames and initials, article title, abbreviated journal name, year, volume and inclusive page numbers. The last names and initials of all the authors up to 6 should be included, but when authors number 7 or more, list the first 6 authors only followed by "et al". Abbreviations for journal names should conform to those used in MEDLINE.
- If citing a website, provide the author information, article title, website address and the date you accessed the information.

- Reference to an article that is in press must state the journal name and, if possible, the year and volume.

Authors are responsible for the accuracy and completeness of their references and for correct text citation.

Examples are given below.

Standard journal article
Chen Z, Fan M, Bian Z, Zhang Q, Zhu Q, Lu P. Immunolocalization of heat shock protein 70 during reparative dentinogenesis. Chin J Dent Res 2000;3:50–5.

Journal supplement
Kaplan NM. The endothelium as prognostic factor and therapeutic target: what criteria should we apply? J Cardiovasc Pharmacol 1998;32(Suppl 3):S78–80.

Journal article not in English but with English abstract
Nakayama H, Ishikawa T, Yamashita S, Fukui I, Mutoh T, Hikichi K, et al. CSF leakage and anosmia in aneurysm clipping of anterior communicating artery by basal interhemispheric approach. No Shinkei Geka 2011;39:263–8. [In Japanese, English abstract]

Book
Bradley EL. Medical and surgical management. Philadelphia: Saunders; 1982, p. 72–95.

Book chapter in book with editor and edition
Greaves M, Culligan DJ. Blood and bone marrow. In: Underwood JCE, editor. General and systematic pathology. 4th ed. London: Churchill Livingstone; 2004, p. 615–72.

Bulletin
World Health Organization. World health report 2002: reducing risk, promoting healthy life. Geneva, Switzerland: World Health Organization; 2002.

Company/manufacturer publication/pamphlet
Eastman Kodak Company, Eastman Organic Chemicals. Catalog no. 49. Rochester, NY: Eastman Kodak; 1977, p. 2–3.

Electronic publications
Duchin JS. Can preparedness for biological terrorism save us from pertussis? Arch Pediatr Adolesc Med 2004;158:106–7. Available from: http://archpedi.ama-assn.org/cgi/content/full/158/2/106. Accessed June 5, 2004.

Smeeth L, Iliffe S. Community screening for visual impairment in the elderly. Cochrane Database Syst Rev 2002(2):CD001054. doi:10.1002/14651858.CD1001054.

Items presented at a meeting but not yet published
Durbin D, Kallan M, Elliott M, Arbogast K, Cornejo R, Winston F. Risk of injury to restrained children from passenger air bags. Paper presented at: 46th Annual Meeting of the Association for the Advancement for Automotive Medicine; September 2002; Tempe, AZ.

Greenspan A, Eerdekens M, Mahmoud R. Is there an increased rate of cerebrovascular events among dementia patients? Poster presented at: 24th Congress of the Collegium Internationale Neuro-Psychopharmacologicum (CINP); June 20–24, 2004; Paris, France.

Khuri FR, Lee JJ, Lippman SM. Isotretinoin effects on head and neck cancer recurrence and second primary tumors. In: Proceedings from the American Society of Clinical Oncology; May 31–June 3, 2003; Chicago, IL. Abstract 359.

Item presented at a meeting and published
Cionni RJ. Color perception in patients with UV- or blue-light-filtering IOLs. In: Symposium on Cataract, IOL, and Refractive Surgery. San Diego, CA: American Society of Cataract and Refractive Surgery; 2004. Abstract 337.

Material accepted for publication but not yet published
Carrau RL, Khidr A, Crawley JA, Hillson EM, Davis JK, Pashos CL. The impact of laryngopharyngeal reflux on patient-reported quality of life. Laryngoscope. In press.

Ofri D. Incidental findings: Lessons from my patients in the art of medicine. Boston, MA: Beacon Press. In press.

Theses and dissertations
Undeman C. Fully automatic segmentation of MRI brain images using probabilistic diffusion and a watershed scale-space approach [master's thesis]. Stockholm, Sweden: NADA, Royal Institute of Technology; 2001.

Ayers AJ. Retention of resin restorations by means of enamel etching and by pins [dissertation]. Indianapolis: Indiana University; 1971.

Website
American Association of Oral and Maxillofacial Surgeons. Wisdom teeth. AAOMS Web site. http://www.aaoms.org/wisdom_teeth.php. Published January 23, 2008. Updated March 9, 2009. Accessed November 15, 2009.

9.7. Tables

Tables should supplement, not duplicate, the text. They should have a concise table heading, be self-explanatory, and numbered consecutively in the order of their citation in the text. Information requiring explanatory footnotes should be denoted using superscripted lowercase letters in alphabetical order (a, b, c, etc.). Asterisks (*, **) are

used only to indicate the probability level of tests of significance. Abbreviations used in the table must be defined and placed after the footnotes. If you include a block of data or table from another source, whether published or unpublished, you must acknowledge the original source.

9.8. *Figures*

9.8.1. General guidelines

The number of figures should be restricted to the minimum necessary to support the textual material. They should have an informative figure legend and be numbered in the order of their citation in the text. All symbols and abbreviations should be defined in the legend. Patient identification should be obscured. All lettering should be done professionally and should be in proportion to the drawing, graph or photograph. Photomicrographs must include an internal scale marker, and the legend should state the type of specimen, original magnification and stain.

Figures must be submitted as separate picture files at the correct resolution (see Section 9.7.2. below). The files should be named according to the figure number, e.g., "Article1_Fig1", "Article1_Fig2".

9.8.2. Formats

Regardless of the application used, when your electronic artwork is finalized, please "save as" or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

- EPS: Vector drawings. Embed the font or save the text as "graphics".
- TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
- TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.
- TIFF: Combination of bitmapped line/half-tone (color or grayscale): a minimum of 600 dpi is required.
- DOC, XLS or PPT: If your electronic artwork is created in any of these Microsoft Office applications, please supply "as is".

Please do not:

- Supply files that are optimized for screen use (like GIF, BMP, PICT, WPG); the resolution is too low;
- Supply files that are too low in resolution;
- Submit graphics that are disproportionately large for the content.

A detailed guide on electronic artwork is available at http://www.elsevier.com/artworkinstructions.

10. The Editorial and Peer Review Process

As a general rule, the receipt of a manuscript will be acknowledged within 1 week of submission, and authors will be provided with a manuscript reference number for future correspondence. If such an acknowledgment is not received in a reasonable period of time, the author should contact the Editorial Office.

Submissions are reviewed by the Editorial Office to ensure that it contains all parts. The Editorial Office will not accept a submission if the author has not supplied all the material and documents as outlined in these author instructions.

Manuscripts are then forwarded to the Editor-in-Chief, who makes an initial assessment of it. If the manuscript does not appear to be of sufficient merit or is not appropriate for the Journal, then the manuscript will be rejected without review.

Manuscripts that appear meritorious and appropriate for the Journal are reviewed by at least two Editorial Board members or expert consultants assigned by the Editor-in-Chief. Authors will usually be notified within 6 weeks of whether the submitted article is accepted for publication, rejected, or subject to revision before acceptance. However, do note that delays are sometimes unavoidable.

11. Preparation for Publication

Once a manuscript has been accepted for publication, the authors should submit the final version of the manuscript in MS Word format, with all tables/figures as applicable, to the Editorial Office.

Accepted manuscripts are copyedited according to the Journal's style and PDF page proofs are e-mailed by the Publisher to the corresponding author for final approval. Authors are responsible for all statements made in their work, including changes made by the copy editor.

12. Publication Charges and Reprints

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CHECKLIST

Only complete manuscript submissions will be considered for publication. Complete submission must include:

- ☐ Cover letter for manuscript submission
- ☐ Authorship statement signed by all authors
- ☐ Signed conflicts of interest disclosure statement
- ☐ Signed copyright transfer agreement
- ☐ Manuscript in MS Word format

AND, where applicable

- ☐ Letter of approval from review committee for use of human samples in research and human experiments
- ☐ Letter of approval from relevant authority for use of animals in experiments
- ☐ CONSORT flow chart for randomized controlled trial
- ☐ Signed consent to publish (in print and online) from human subjects who can be identified in your manuscript
- ☐ Letter(s) of permission from copyright holder(s) to use copyrighted sources in your manuscript

In the actual article, ensure that the following information is provided:

- ☐ Title page
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  - ○ Article title
  - ○ Name(s) and affiliation(s) of author(s)
  - ○ Corresponding author details (name, e-mail, mailing address, telephone and fax numbers)
- ☐ Abstract: structured for Original Article; unstructured for Review Article, Case Report, Short Communication (none required for Editorial, Letter to the Editor)
- ☐ 3–5 relevant keywords in alphabetical order: required for Review Article, Original Article, Case Report, Short Communication (MeSH terms are recommended; see http://www.ncbi.nlm.nih.gov/mesh?term)
- ☐ Main text
- ☐ References in the correct format, cited in numerical order, and all references in the List are cited in the Text/Tables/Figures, and vice versa

AND, where applicable

- ☐ Acknowledgments
- ☐ Conflicts of interest statement
- ☐ Table headings and tables, each on a new page
- ☐ Figure legends, on a new page
- ☐ Electronic picture files of all figures; resolution of 300 dpi for halftone images, 600 dpi for combination art (halftone + line art), and 1000 dpi for line art

Further considerations:

- ☐ Manuscript has been spell-checked and grammar-checked
- ☐ Color figures are clearly marked as being intended for: (I) color reproduction on the Web (free of charge) and in print; or (II) color reproduction on the Web (free of charge) and in grayscale in print (free of charge). If option (II), then grayscale versions of the figures are also supplied for printing purposes.

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All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in *BioMedicine*

Authorship contributions

Please indicate the specific contributions made by each author (list the authors' initials followed by their surnames, e.g., Y.L. Chang). The name of each author must appear at least once in each of the three categories below.

Category 1

Conception and design of study: _____, _____, _____, _____;

acquisition of data: _____, _____, _____, _____;

analysis and/or interpretation of data: _____, _____, _____, _____.

Category 2

Drafting the manuscript: _____, _____, _____, _____;

revising the manuscript critically for important intellectual content: _____, _____,

_____, _____.

Category 3

Approval of the version of the manuscript to be published (the names of all authors must be listed):

_____, _____, _____, _____, _____, _____,

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Acknowledgments

All persons who have made substantial contributions to the work reported in the manuscript (e.g., technical help, writing and editing assistance, general support), but who do not meet the criteria for authorship, are named in the Acknowledgments and have given us their written permission to be named. If we have not included an Acknowledgments, then that indicates that we have not received substantial contributions from non-authors.

This statement is signed by all the authors (*a photocopy of this form may be used if there are more than 10 authors*):

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Author names:

This statement is signed by all the authors to indicate agreement that the above information is true and correct (*a photocopy of this form may be used if there are more than 10 authors*):

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