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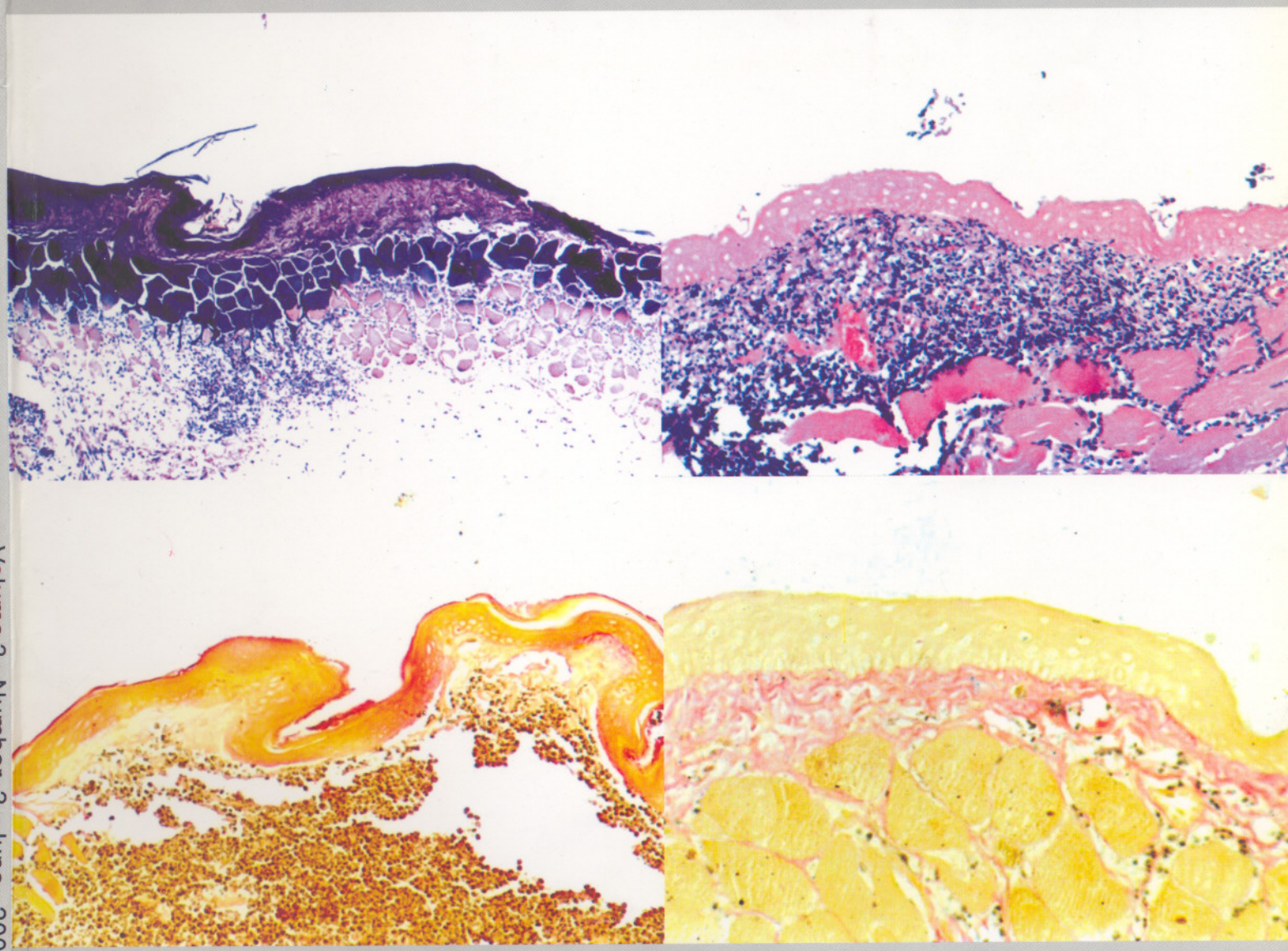

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CONTENTS

On the cover : Hamster's cheek pouch tissues after treated with Carnoy's solution and TCA, respectively.

The left top one showed the necrosis of epithelia and penetration into muscular layer after treated with Carnoy's solution for one week, with HE staining. The right top one showed disappearance of cellular structure, and the vacuolar shadow of the cells after treated with 35% TCA for one week, with HE staining. The left bottom one showed disordered arrangement of collagen fibers, the broken or even disappeared collagen fibers after treated with Carnoy's solution for one week, with Van Gieson staining. The right bottom one showed disappearance of fibroblast after treated with 20% TCA for one week, with Van Gieson staining. See *Effects of Carnoy's Solution and TCA on the Pouch Mucosa of Hamster* by Xinguang Han et al, page 35 - 40 in this issue.

DNA Methylation and Esophageal Squamous Cell Carcinoma: Special Reference to Research in China

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Abstract: Genetic abnormalities of proto-oncogenes and tumor suppressor genes have been demonstrated to be involved frequently in esophageal carcinogenesis, especially the hypermethylation of CpG islands. Accumulated evidences indicate that hypermethylation of CpG islands in the promoter regions are one of the important mechanisms to silence the expression of many important genes and may play an important role in esophageal carcinogenesis. In this review, evidences for gene hypermethylation in human esophageal precancerous and cancerous lesions with special reference to research in China and their correlations with other populations in Asia were summarized to provide molecular clues for identifying the biomarkers for high-risk subject screening and early diagnosis. [Life Science Journal. 2006;3(2):1-11] (ISSN: 1097-8135).

Keywords: methylation; esophageal squamous cell carcinoma; carcinogenesis; CpG islands

Abbreviations: APC: adenomatous polyposis coli; BCH: basal cell hyperplasia; CIS: carcinoma in site; CRBP1: cellular retinol-binding protein 1; DNMT: DNA methyltransferase; DYS: dysplasia; EAC: esophageal adenocarcinoma; LOH: loss of heterozygosity; MGMT: O^6-methylguanine-DNA methyltransferase; NSCLC: non-small cell lung cancer; RARβ2: retinoic acid receptor-beta 2; RASSF1: Ras-association domain family 1; SCC: squamous cell carcinoma; TIG1: tazarotene-induced gene 1; TSG: tumor suppressor genes; TSLC: tumor suppressor in lung cancer

1 Introduction

Esophageal cancer is one of the most common malignant diseases, with a remarkable geographical distribution and poor prognosis[1]. The five-year survive rate is only 10%. However, the five-year survive rate for the patients with the early esophageal cancer is more than 90%. But in clinical, more than 85% of the esophageal cancer patients are diagnosed at the late stage. Lack of early specific symptoms and diagnosis biomarker remains the leading cause of late diagnosis for esophageal cancer. Therefore, the current challenges in esophageal cancer research are to obtain a better understanding of the underlying molecular alterations to establish the strategies for high-risk subject screening and early diagnosis. Cancer of the esophagus exists in two main forms with different etiological and pathological characteristics, i. e. esophageal squamous cell carcinoma and esophageal adenocarcinoma[2]. It has been well recognized that esophageal carcinogenesis is a multistage and progressive process with a sequence of from basal cell hyperplasia to dysplasia to carcinoma in site and esophageal carcinoma. A variety of genetic lesions has been demonstrated to be involved in esophageal carcinogenesis, including p53-Rb pathway with gene amplifications, loss of heterozygosity or homozygous deletions, point mutations, and chromosomal rearrangements[2-4]. Besides, the accumulated evidences in the epigenetic inactivation of genes have shown their importance in esophageal carcinogenesis, as important a driving force as the inactivation of genes by mutation[5].

"Epigenetic" events, i.e. heritable changes in gene function which can not be explained by changes in DNA sequence, are composed of histone acetylation, the chromatin structure and DNA methylation[5]. DNA methylation seems to be the most important mechanism for "epigenetic change" at present[5,6]. Through a process of covalent modification catalyzed by DNA methyltransferase, the DNA of mammalian cells contains a "fifth base", namely 5-methylcytosine. The most frequent target for this modification is cytosine in the context of the dinucleotide CpG[5]. Throughout the genome CpG dinucleotides are found at one-fifth of their

predicted frequency[7]. In marked contrast to the genome wide under representation of CpGs, there are regions of the genome termed CpG islands which have maintained their expected frequency of this dinucleotide. And the CpG islands are often found within the promoter of the genes[7,8]. There is an inverse relationship between the density of promoter methylation and the transcriptional activity of a gene[9,10]. The mechanism of gene silencing by promoter hypermethylation has recently been shown to be related with the recruitment of repressor protein complex, resulting in deacetylation of the chromatin and histone, thus barring access of the active transcription complex. However, the actual mechanisms by which DNA methylation modulates gene expression are largely unknown[11,12]. The assays for detection of cytosine methylation could be divided into two groups: restriction enzyme-based and bisulfite treatment-based[13,14]. The former employs the inhibition of certain restriction enzymes by methylation of their recognition sites as an indicator for the presence of methylation. The latter translates the epigenetic information of cytosine methylation in primary sequence differences by converting unmethylated cytosine to uracil whereas methylated cytosine remains unaltered. The bisulfite-converted genomic DNA can be analyzed by a wide variety of PCR-based methods of which direct sequencing of the PCR products or sequencing of individual PCR product clones gives the most detailed information about the methylation pattern in the CpG islands under study[14,15].

Methylation is needed for the normal development of cells. Genome stability and normal gene expression are largely maintained by a fixed and predetermined pattern of DNA methylation[15]. Aberrant methylation confers a selective growth advantage that results in cancerous growth[15]. From various lines of evidence, it is known that the methylation pattern of the cancerous cell is associated with a broad genomic hypomethylated state that is often accompanied by a more regional and locus-specific hypermethylated pattern[7]. The presence of alterations in the profile of DNA methylation in cancer was initially thought to be exclusively a global hypomethylation of the genome that would possibly lead to massive overexpression of oncogenes whose CpG islands were normally hypermethylated[14]. Nowadays, however, this is considered to be an unlikely or, at best, incomplete scenario[14]. The popularity of the concept of demethylation of oncogenes leading to their activation is in clear decadency[14,15]. Hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes is now firmly established as an important mechanism for gene inactivation[16-18].

The particular genes that are hypermethylated in tumor cells are strongly specific to the tissue of origin of the tumor. A profile of CpG island hypermethylation exists according to the tumor type[15]. The mechanism responsible for this type of pattern remains unclear. Moreover, accumulating evidence indicates that CpG island hypermethylation is an early event in cancer development and, in some cases, may precede the neoplastic process[19]. Therefore, such profiles would provide invaluable insight into mechanisms underlying the evolution of each tumor type and will provide new molecular markers. This review will focus on the current understanding of DNA methylation abnormalities in esophageal cancer and provide molecular clues for identifying the biomarkers for high-risk subject screening and early diagnosis.

2 Studies of Genes Promoter Hypermethylation in SCC

Table 1 complied genes[20-55] that have been extensively studied in the past with a special reference to Chinese population, especially for those from Linxian, the highest incidence area for esophageal cancer in Henan, northern China. Putative TSG, involving apoptosis, cell adherence, DNA repair, and the cell cycle, have been investigated for hypermethylation by various techniques in esophageal cancer.

2.1 $p14^{ARF}$, p15 and p16

The 9p21 chromosomal band is one of the most frequently altered genomic regions in human cancers[56]. Within a short distance of 50 kb, a gene cluster consisting of three genes, $p14^{ARF}$, p15 and p16, are harbored. All of which have putative tumor suppressor roles[56-58]. Inactivation of $p14^{ARF}$, p15 and p16 genes has been observed in many types of human cancers including SCC[20,22,26,57,58]. For example, our results from immunohistochemical analysis indicated that p16 expression was present in only 3 out of 22 SCC cases[59]. Some studies showed that germline mutations in p16 gene might be related to familial melanoma[60], but our study found the mutation of p16 gene in esophageal cancer was rare[20]. Hemizygous and homozygous deletion at 9p21 are widely considered to be one of the primary mechanisms of p16/p15 inactivation[20]. Recently, however, aberrant methylation of the CpG island at the pro-

moter regions of p16 and p15 genes was reported in SCC and was associated with loss of transcription[20-31]. By analyzing the $p14^{ARF}$, p15 and p16 genes individually in 40 SCC, we detected aberrant promoter methylation of the p16 gene in 16 (40%), of $p14^{ARF}$ in 6 (15%), and of p15 in 5 (12.5%) tumor samples. We further detected homozygous deletion of p16 in 7 (17.5%), of $p14^{ARF}$ in 13 (33%), and of p15 in 16 (40%) tumor samples, and detected no mutation in the $p14^{ARF}$ and p16 genes[20, 26]. The above results suggest that $p14^{ARF}$, together with p15, is a primary target of homozygous deletion, whereas p16 is the hypermethylation hotspot in human esophageal cancer. Johanna *et al* analyzed methylation of CDKN2A ($p16^{INK4\alpha}$ and $p14^{ARF}$) individually in 40 SCC from Linxian, and detected aberrant promoter methylation of the $p16^{INK4\alpha}$ gene in 4 (19%), of $p14^{ARF}$ in 11 (52%), in 21 tumor samples[22]. Although samples in two studies above collected were all from the same area, the results were different markedly. The difference probably results from: 1) Regions for the patients studied are not completely the same. In Johanna's study, part of the SCC patients was from Linzhou; in the other two studies, all the SCC patients were from Linzhou. 2) Different methylation primes and the different sites to be detected. The 5′ position of the sense unmethylated and methylated primers of $p14^{ARF}$ gene corresponds to bp 227 and 225 of GenBank sequence number L41934 in our study, which respectively amplify a 165-bp and 160-bp product[22,26]. However, the 5′ position of the sense unmethylated and methylated primers of $p14^{ARF}$ gene corresponds to bp 195 and 201 of GenBank sequence number L41934 in the latter, which respectively amplify a 132-bp and 122-bp product[22,26]. 3)Difference in histopathological types of collected samples. In our study, pathology grades of the samples enrolled were unknown. But in the latter, the majority tumors analyzed were of Ⅰ-Ⅱ grade (15/21), and 4 cases belonged to grade Ⅲ, for two cases grades were not listed[20]. 4) Different criteria in the process of enrolling samples. In Yan's study, since DYS and CIS are pathologically similar, CIS were combined into group DYS[26]. Guo *et al* analyzed methylation of p16 gene in 37 SCC from Hebei, and detected aberrant promoter methylation of the p16 gene in 21 (64.9%)[29]. The similar results were observed for Japanese people[23-25,28]. The p16 methylation in SCC was higher in Japanese people than in Chinese people, suggesting the possibility of different carcinogenic factors and mechanisms involved in different populations.

2.2 The FHIT gene

The FHIT gene is located at chromosome 3p14.2 and encodes a polypeptide of 147 amino acids[61]. FHIT allelic deletions and reduced or absent FHIT protein expression have been observed in a variety of tumors suggesting a putative tumor suppressor function[61, 62]. In SCC, the hypermethylation of CpG island in the FHIT promoter region was significantly correlated with the deletion of FHIT protein expression[26,29,42,47,52]. Methylated SCC cell lines exhibit re-expression of the FHIT gene and demethylation in the CpG island after treatment with demethylating agent 5-aza-2′-deoxycytidine[63]. These findings suggest that methylation of the 5′ CpG island of the FHIT gene is closely associated with transcriptional inactivation and might be involved in tumor development of the esophagus.

2.3 The RARβ2 gene

The retinoic acid receptor-beta 2 gene located at 3p24 has been intensively studied in many cancers and found to have defective function, thus making it a candidate TSG[64]. We found that $RAR\beta_2$ was detected in 36% (18/50) of normal esophageal tissues, and that 14 of 20 (70%) SCC samples had hypermethylation of the $RAR\beta_2$ promoter[40]. Another group of study reported that 27 of 47 (55%) primary resected SCC samples showed $RAR\beta_2$ methylation[42]. Liu *et al* analyzed the methylation status of $RAR\beta_2$ promoter region and its expression in 51 SCC tissue samples with their adjacent normal epithelia and two normal esophageal epithelia, and found that there was a statistically significant correlation between methylation status of $RAR\beta_2$ and tumor grade[33]. Moreover, a relationship between methylation status and decreased $RAR\beta_2$ expression was found only in G (2) stage tumors. Methylation of $RAR\beta_2$ promoter regions was detected in 26/51 (51%) of the primary tumors; moreover in seven tissue samples with their adjacent normal epithelia, methylation of this locus was found in both the adjacent normal epithelia and matched tumor tissues; and in other 19 tissue samples methylation of this locus only existed in the primary esophageal tumors; and in two cases, hypermethylation was observed in the adjacent normal epithelia, but not in the corresponding SCC samples[33]. Thus, they considered that $RAR\beta_2$ methylation is a common neoplastic feature of SCC. These results identified methylation as the underlying mechanism for this frequent loss of $RAR\beta_2$ in esophageal cancer[33,34,39,40,42].

Table 1. Compilation of genes hypermethylated in SCC by regions and methods

Genes	Regions for the patients studied	Incidence of Methylation		Methods for methylation	References
		Cancer tissue *n/N* (%)	ANT# *n/N* (%)		
p14ARF	Linxian (T#)	6/40(15%)	ND#	MSP, Sequencing	20
	Linxian (T)	11/21(52%)	ND	MSP, HPLC	22
	Linxian (T)	6/21(28.6%)	10/40(25%)	MSP	26
p15	Linxian (T)	5/40(12.5%)	ND	MSP, Sequencing	20
	Linxian (T)	2/25(8%)	3/75(4%)	MSP	26
p16	Linxian (T)	16/40(40%)	ND	MSP, Sequencing	20
	Cell lines	4/30(13%)	ND	MSP	21
	Linxian (T)	4/21(19%)	ND	MSP, HPLC	22
	Japan (T)	6/31(19%)	ND	Sequencing	23
	Japan (T)	30/42(71.4%)	24/30(80%)	MSP	24
	Japan (T)	31/38(82%)	ND	MSP	25
	Japan (S#)	7/31(23%)	ND	MSP	25
	Linxian (T)	7/21(33.3%)	12/40(30%)	MSP	26
	Sporadic (T)	5/34(15%)	ND	MSP in situ	27
	Iran (T)	22/30(73.3%)	ND	MSP	28
	Iran (B#)	13/30(43.3%)	ND	MSP	28
	Iran (S)	8/30(26.6%)	ND	MSP	28
	Iran(family)(B)	18/28(64.3%)	ND	MSP	28
	Hebei (T)	21/37(64.9%)	5/79(6.3%)	MSP	29
	Hubei (S)	34/56(61%)	ND	nMSP	30
		15/56(27%)		MSP	
NMDAR2B	USA(T) and cell lines	95%	ND	MSP	31
RASSF1A	Hong Kong (T)	22/64(34%)	3/64(5%)	MSP	32
	Cell lines	3/7(43%)		Sequencing	
	Japan (T)	24/47(51%)	2/47(4%)	MSP	32
	Cell lines	16/22(73%)			
	Japan (T)	25/48(52%)	ND	MSP	36
	Cell lines	17/23(74%)			
	Japan (T)	13/55(24%)	ND	MSP	73
RARβ_2	Linxian and Anhui (T)	26/51(51%)	9/51(18%)	MSP	33
	Cell lines	4/6(67%)		MSP	39
	Linxian (T)	14/20(70%)	18/50(36%)	MSP	40
	Japan (T)	7/28(25%)	1/10(10%)	MSP	34
	Japan (T)	27/47(55%)	18/47(38%)	MSP	42
CRBP1	Japan (T)	5/28(17.9%)	0/28(0)	MSP	34
TIG1	Japan (T)	5/28(17.9%)	0/28(0)	MSP	34
MGMT	Linxian (T)	13/18(72%)	23/49(47%)	MSP	35
	Sporadic (T)	46/119(38.7%)	5/22(22.7%)	MSP	43
hMLH1	Japan (T)	6/9(66.7%)	ND	MSP	36
	Japan (T)	0/30(0)	ND	MSP	45
	Linxian (T)	5/25(20%)	7/75(9%)	MSP	26
	Sporadic (T)	30/92(32.6%)	ND	MSP	53
E-cadherin	Japan (T)	25/41(61%)	ND	MSP	37

	Linxian (T)	4/25(16%)	0/75(0)	MSP	26
	Hong Kong (T)	16/20(80%)	ND	MSP	49
LRP1B	Japan (T)	5/34(14.7)	ND	Bisulfite-PCR Sequencing	38
Trypsin	USA(T)	5/10(50%)	ND	MSP	41
TSLC1	Japan (T)	28/56(50%)	ND	MSP	68
VHL	Japan (T)	6/47(13%)	0/47(0)	MSP	42
	Linxian (T)	2/25(8%)	0/75(0)	MSP	26
FHIT	Japan (T)	21/47(45%)	14/47(30%)	MSP	42
	Japan (T)	25/36(69.4%)	ND	MSP	47
	Linxian (T)	5/25(20%)	3/75(4%)	MSP	26
	Japan (T)	5/35(14%)	0/35(0)	MSP	52
	Hebei (T)	25/37(67.6%)	3/79(3.8%)	MSP	29
ECRG4	Linxian (T)	12/20(60%)	3/20(15%)	MSP, DHPLC	44
HLA class I	Linxian (T)	19/29(66%)	0/29(0)	MSP	50
HLA-A	Linxian (T)	2/25(4%)	0/75(0)	MSP	26
	Linxian (T)	7/29(24%)	0/29(0)	MSP	50
HLA-B	Linxian (T)	5/25(20%)	5/75(7%)	MSP	26
	Linxian (T)	11/29(38%)	0/29(0)	MSP	50
HLA-C	Linxian (T)	5/25(20%)	2/75(3%)	MSP	26
	Linxian (T)	9/29(31%)	0/29(0)	MSP	50
Chfr	Japan (T)	7/43(16.3%)	ND	MSP	48
	Cell lines	4/15(26.7%)			
APC	USA(T)	16/32(50%)	ND	MSP	51
	USA(P#)	2/32(6.3%)			
MT3	Hebei (T)	10/47(21.3%)	ND	COBRA	54
GATA-4	Henan (T)	27/44(61%)	0/44(0)	MSP	55

#ANT, adjacent nonmalignant tissue; ND, not done; T, tumor tissue; S, serum sample; B, blood sample; P, plasma sample

Retinoic acid receptor-beta, cellular retinol-binding protein 1, and tazarotene-induced gene 1 have been linked to retinoic acid signaling. Little is known about the involvement of these three genes in SCC. Mizuiri *et al* found that DNA hypermethylation of RARβ_2 existed in seven (25.0%) of the 28 SCC, of CRBP1 in five (17.9%), and of TIG1 in five (17.9%). DNA methylation of RARβ_2 was identified in one of 10 samples of corresponding non-neoplastic mucosa (10.0%), whereas no DNA methylation of CRBP1 or TIG1 was detected. No correlation was found between the DNA methylation status of RARβ and clinicopathological factors such as depth of invasion, lymph node metastasis, or tumor stage. In contrast, DNA methylation of both CRBP1 and TIG1 was observed only in stage Ⅲ SCC[34]. These results showed that inactivation of the retinoic acid signaling-associated genes RARβ_2, CRBP1, and TIG1 by DNA methylation occurred frequently in SCC.

2.4 The APC gene

The adenomatous polyposis coli gene, located on chromosome 5q21, is a TSG in the WNT signaling pathway[65]. We found that APC shows frequent LOH in esophageal carcinomas, and the prevalence of mutations in the APC gene in esophageal carcinomas is low. Hypermethylation of the promoter region of the APC gene occurred in abnormal esophageal tissue in 16 (50%) of 32 patients with SCC, but not in matching normal esophageal tissues[51]. So methylation of the promoter region of this gene constitutes an alternative mechanism of gene inactivation in esophageal carcinoma.

2.5 The MGMT gene

The human enzyme O^6-methylguanine-DNA methyltransferase protects the cell from guanine methylation by irreversibly transferring the alkyl group of the O^6-methylguanine to a specific cysteine residue within the molecule[66]. Approximately 20% of tumor cell lines lack MGMT activity and

are highly sensitive to alkylating agents[66,67]. In established cancer cell lines, MGMT expression appears to be correlated with methylation in the promoter of the gene[35,66]. The development of SCC has been linked to exposure to carcinogens such as nitrosamines that cause various alkyl DNA damages and MGMT is a primary defense against alkylation-induced mutagenesis and carcinogenesis. We found that 18 (72%) SCC samples had DNA hypermethylation in the MGMT promoter region, and that the frequency of the loss of MGMT mRNA and protein expression was highly correlated with MGMT promoter hypermethylation according to Fisher's exact tests[35]. The gene has been shown to be methylated in 46/119 SCC (38.7%), but all 21 normal esophageal tissues had unmethylated MGMT[43].

2.6 The E-cadherin gene

E-cadherin is a *Mr* 120,000 transmembrane glycoprotein expressed on the surface of epithelial cells. In epithelial tissues, E-cadherin mediates homophilic, Ca^{2+}-dependent intercellular adhesion that is essential for the maintenance of normal tissue architecture[49]. Loss of E-cadherin expression occurs in a variety of human tumors and is correlated with invasion and metastasis, and activation of E-cadherin results in the growth inhibition of tumor cell lines[37]. E-cadherin can be targeted by both genetic and epigenetic means. Moreover, the hypermethylation of E-cadherin was seen frequently in most tumor types, but mutations only frequent in a small number of specific subtypes[37]. E-cadherin, a cell adhesion molecule, is regarded as an invasion-suppressor molecule and a prognostic marker in many types of human cancers. In esophageal carcinoma, downregulation of E-cadherin is common and is associated with an increase in invasive and metastatic potential, but mutations of the gene are rare[49]. E-cadherin was methylated in 16 of 20 (80%) SCC samples in Hong Kong people and 4 of 6 SCC cell lines[49]. And treatment of E-cadherin negative carcinoma cells with the demethylating agent, 5-aza-2′-deoxycytidine, induced re-expression of the gene[49]. In Japan, E-cadherin was methylated in 25 of 41 (61%) SCC samples[37]. However, we found that E-cadherin was methylated in 4 of 25 (16%) SCC clinical samples in Linxian, Northern China[26]. These data suggest that epigenetic silencing via aberrant methylation of the E-cadherin promoter is the critical mechanism for inactivation of this gene in esophageal cancer, and that the frequency of the hypermethylation of E-cadherin varied with the patients from different regions.

2.7 The TSLC1 gene

Tumor suppressor in lung cancer was first characterized as a TSG in human non-small cell lung cancer and termed TSLC1[68]. The tumor suppressor role of this gene has been demonstrated in the cell lines of NSCLC, hepatocellular carcinoma, pancreatic cancer and SCC[68,69]. Loss of TSLC1 expression was observed in 75% of the SCC cell lines and 50% of the primary tumors from SCC patients[69]. Kaganoi *et al*[68] examined the methylation status of six cytosine residues of CpG sites in a putative promoter sequence upstream from the translation initiation site by bisulfite sequencing in four cell lines, including KYSE270, which expressed TSLC1, and KYSE410, KYSE520, and KYSE960, which did not express it, and found that all of the cytosine residues in KYSE270 DNA were unmethylated, whereas all of the six cytosine residues in KYSE520 DNA and five residues in KYSE410 and KYSE960 DNA were methylated. Especially, the cytosine residues in KYSE520 DNA were all hypermethylated.

2.8 The RASSF1A gene

Many known Ras effectors are oncoproteins on their own. Less is known about Ras effectors possessing tumor suppressor properties[42]. Recently, a new family of genes encoding a putative Ras effector, the Ras-association domain family 1 gene, has been identified within the critical lung and breast cancer deletion region at 3p21.3. The RASSF1 locus encodes several major transcripts by alternative promoter selection and alternative mRNA splicing: RASSF1A, RASSF1B and RASSF1C. Many studies have suggested that RASSF1A was a new putative TSG[32, 42,46]. RASSF1A acts as a negative effector of Ras in a pro-apoptotic signaling pathway. Interestingly, mutational inactivation of this gene is very rare (< 2%), and the main mechanism of its inactivation is through promoter methylation and LOH[46]. The RASSF1A isoform is highly epigenetically inactivated in lung, breast, ovarian, kidney, prostate, thyroid, esophagus and several other carcinomas[32]. Hypermethylation of RASSF1A was detected in 73% of SCC cell lines and 51% of primary SCC from Japan, whereas only 4% of RASSF1A hypermethylation were detected in corresponding noncancerous tissues[42]. There was a statistically significant correlation between the presence of hypermethylation and tumor stages[27]. Hypermethylation of RASSF1A was found in 3/7 (43%) of SCC cell lines and 22/64 (34%) of primary SCC from Hong Kong peoples[32]. These findings sug-

gest that epigenetic silencing of RASSF1A gene expression by promoter hypermethylation could play an important role in SCC carcinogenesis.

Besides the above mentioned genes, there are hypermethylation of some other genes involved in esophageal cancer, including hMLH1 (0 – 66.7%)[26,36,45,53], VHL (8 – 13%)[26,42], MT3 (21.3%)[54], NMDAR2B (95%, USA)[31], LRP1B (14.7%, Japan)[38], Trypsin (50%, USA)[41], ECRG4 (60%)[44], GATA-4 (61%)[55], Chfr (16.3%, Japan)[48] and HLA class I[26,50] including HLA-A (4 – 24%), HLA-B (20 – 38%) and HLA-C (20 – 31%). Because of the limited case number and low frequency of methylation or the populations outside Asia, the discussion was not expanded on these studies.

3 Methylation in Serum from the Esophageal Cancer Patients

DNA methylation of the promoter region of certain cancer-associated genes is one potential early detection biomarker[25,30,51]. Genetic analysis has shown that cell-free circulating DNA in plasma or serum of cancer patients share similar genetic alterations to those described in the Table 1[25,28,30,51]. Numerous studies have demonstrated the presence of promoter hypermethylation of tumor suppressor genes in the serum DNA of patients with various cancers[30]. Hypermethylated APC DNA was observed in the plasma of two of 32 (6.3%) SCC[51]. Hibi *et al*[25] found that aberrant promoter methylation of the p16 gene was detected in 31 of 38 (82%) SCC, and 7 of these 31 (23%) patients with a p16 alteration in the primary tumor had the same methylation changes in the corresponding serum DNA. This study yielded a promising result: a tumor associated DNA alteration could be detected in the serum of 18% of SCC patients (7 of 38 patients) using p16 methylation as a target. Moreover, the clinical sensitivity of this assay can be potentially improved by incorporating other possibly methylated target genes, which has been estimated in other tumor types. For example, Fukami *et al*[69] analyzed primary NSCLC and serum from 22 patients for the methylation pattern of four TSG (DAPK, GSTP1, p16 and MGMT). Methylation of at least one of these genes was detected in 68% of NSCLC. Comparing primary tumors with methylation and matched serum samples, 73% of the matched serum samples were found to be methylation. In addition, none of the sera from the patients with tumors not demonstrating methylation was positive[69]. Yao *et al* detected 61% p16 methylation in 56 serum samples of SCC by nMSP. In contrast, 27% p16 methylation in 56 serum samples of SCC was detected by MSP[30]. Therefore, combined detection of aberrant promoter hypermethylation of cancer-related genes in serum may be useful for esophageal cancer diagnosis or the detection of recurrence. Improved method to detect methylation would increase sensitivity.

3.1 Early aberrant DNA methylation in esophageal carcinogenesis

In many tumors, it has been proved that aberrant DNA methylation frequently occurs in precancerous tissue as well as cancer tissue, and both factors, genetic and epigenetic, lie at the origin of carcinogenesis[59]. The relative contribution of each varies significantly in different human tumors[59]. In our previous report, we compared hypermethylation of p16, p15, p14, HLA-A, -B, -C, hMLH1, E-cadherin, FHIT and VHL genes in SCC tumor, neighboring normal and precancerous tissues. We found that in 48 biopsy samples with BCH or DYS, the most frequent hypermethylated genes were p16 (18.8%) and $p14^{ARF}$ (14.6%), and seventeen out of these 48 samples (35.4%) contained hypermethylation of at least one gene[26]. In the resected tissue samples, 52% of the BCH and 81% of the tumors showed hypermethylation of at least one gene. Moreover, genes hypermethylated in earlier stage lesions were always found hypermethylated at the later stage lesions in the same patient[26]. In another study, we reported that two of 17 (12%) normal, 9 of 21 (43%) BCH, 7 of 12 (58%) DYS, and 14 of 20 (70%) SCC samples had hypermethylation of the $RAR\beta_2$ promoter region[40]. As to progression of EAC, it has been reported that, methylation of the p16 promoter was detected in 18 of 22 (82%) EAC and 10 of 33 (30%) premalignant lesions, whereas no methylation of the p16 promoter was found in normal esophageal epithelia[59]. These data suggest that aberrant DNA methylation participates early in the development of esophageal cancer. Recent studies showed that epigallocatechin-3-gallate (EGCG), the major polyphenol from green tea, inhibited DNMT activity and reactivate several methylation-silenced genes, including p16, $RAR\beta_2$, MGMT and hMLH1, in human esophageal cancer KYSE 510 cells, accompanied by the expression of mRNA of these genes[70]. The result suggests that methylation might be a new target of chemopreventive activity. In the last two decades, it has been proved that many drugs, such as tamoxifen, aspirin, COX-2 inhibitors, possess positive chemopreventive

activity against esophageal cancer[4]. However, the related mechanisms have not been elucidated so far. Therefore, it will be very attractive to examine the effect of these drugs on promoter methylation status of key genes in esophageal precancerous lesions.

3.2 Hypermethylation as a target of therapeutic intervention

It has been reported that demethylating agents 5-azacytidine and 5-aza-2′-deoxycytidine can reactivate the demethylated state of several TSG and increase their expression in various cancers, including esophageal cancer, *in vitro* and *in vivo*[21,22,33,35,71,72]. Since methylation and transcriptional status are inversely correlated, the use of demethylating agents appears to be a promising option for the treatment of tumors. Methylation of genes in tumor cells could provide a tumor specific target for new therapies[33,35,71]. In fact, these demethylating agents have exhibited significant activity in the treatment of patients with myelodysplastic syndrome, chronic myeloid leukaemia and acute myeloid leukaemia[24,71]. However, preliminary experience with these agents in solid tumors has been relatively poor[20,33]. Esophageal tumor shows a high prevalence of TSG hypermethylation, and the above studies demonstrated that gene expression could be restored after treatment of esophageal tumor cells with demethylating agents *in vitro*. However, up to date the clinical trial about demethylating agents in esophageal cancer is unavailable. Although it is too early to make any expectation about the effect of these drugs on esophageal cancer, this is a very promising concept and needs to be tested in further studies[72].

3.3 Significance of methylation in clinical application

Emerging evidence suggests a possible prognostic value of gene promoter hypermethylation. Kawakami *et al*[51] reported that high plasma levels of methylated APC DNA were statistically significantly associated with reduced patient survival. Schulmann *et al*[74] analyzed the methylation status of ten genes (HPP1, RUNX3, RIZ1, CRBP1, 3-OST-2, APC, TIMP3, p16, MGMT, p14) of 77 EAC samples and found that DNA methylation of some genes individually showed only trends toward diminished survival, whereas patients whose tumors had $>50\%$ of their gene profile methylated had both significantly poorer survival and earlier tumor recurrence than those without positive methylation. The data suggest that combined detection of methylation status for multiple genes is a effective strategy to predict esophageal tumor behavior, to help staging esophageal cancer, to detect recurrent disease, and to monitor disease progression or treatment response. Although some genes that are frequently inactivated by methylation and are of prognostic impact for esophageal cancer patients have already been found, additional genes need to be identified. Thus, patients with a worse prognosis could be selected. These patients might benefit from a more aggressive treatment strategy.

Unlike genetic changes, epigenetic changes could potentially be reversed using DNA methyltransferase inhibitors such as 5-aza-dc in cancer. Clinically, 5-aza-dc and its analogs have been used to treat leukemia and lung cancer[75, 76]. The clinical benefit observed has been associated with the restoration of previously silenced genes. Additionally, one potential advantage of using gene methylation as a biomarker is the fact that its presence or absence is easily established by use of MSP. Hypermethylation biomarkers, in combination with other molecular markers, such as p53 mutation, for predicting early outset of SCC would be valuable for a follow-up study in high-risk area for esophageal cancer.

4 Conclusion and Perspectives

Esophageal carcinogenesis is a multistep process of accumulation of genetic and epigenetic abnormalities. It has become clear that promoter hypermethylation of TSG are important for this progressive process. The steadily growing list of genes inactivated by promoter hypermethylation in esophageal carcinoma provides not only new insights into the molecular basis of the diseases but also a long list of interesting candidate genes for the development of molecular biomarkers for high-risk subject screening and early diagnosis. In addition, the fact that methylation could be reversed *in vitro* raise a new treatment strategy for esophageal cancer treatment and prevention. It is much desirable to develop methylation biomarker from cell free circulating blood samples which is of apparent significantly in large-scale mass survey for high-risk subject screening in the high-risk areas for esophageal cancer. Furthermore, It is crucial to establish standard method in methylation detection to make a fundamental conclusion.

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Expression of Nucleostemin Gene in Human Acute Leukemic Cells

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Abstract: Objective. To investigate the expression of nucleostemin gene in acute leukemic cells and its link to the pathogenesis of acute leukemia. **Methods.** Specific primers were designed according to the consensus sequence of three NS gene variants. Reverse transcriptase PCR was used to detect the expression level of NS gene in two leukemic cell lines (K562 and HL60), three types (acute myeloblastic leukemia (AML), acute monocytic leukemia (AM_0L) and acute lymphoblastic leukemia (ALL)) of primary leukemic cells and bone marrow mononuclear cells (BMMNC) from healthy individuals or patients with benign anemia. β-actin was used as the reference gene. **Results.** NS gene expression were easily detected in K562, HL60, and three groups of leukemic cells, the PCR product intensity ratio of NS gene to β-actin gene in K562, HL60, M1 + M2a, M3, M5a, M5b, and ALL was 0.735 ± 0.26, 0.449 ± 0.19, 0.687 ± 0.21, 0.408 ± 0.16, 0.866 ± 0.27, 0.448 ± 0.19, 0.403 ± 0.19, respectively, but the expression of this gene in the BMMNC of healthy individuals or patients with benign anemia were either not detected or too low to be calculated. In myeloid leukemia, the NS gene expression in leukemic cells at early stage of differentiation was significantly higher than the NS gene expression in leukemic cells at later stage of differentiation (K562 > HL60, M1 + M2a > M3, M5a > M5b, $P < 0.01$). **Conclusions.** NS gene is over-expressed in acute leukemic cells, and the expression level in leukemic cells at different differentiation stage is different. The results suggested (1) NS gene expression closely related to the origination and development of leukemia; (2) cancer cells and the stem cells had similarity in some aspects; and (3) NS gene might be a potential target in the treatment of leukemia. [Life Science Journal. 2006;3(2):12-16] (ISSN: 1097-8135).

Keywords: nucleostemin; leukemia; stem cell; proliferation; differentiation; cell cycle

Abbreviations: ALL: acute lymphoblastic leukemia; AML: acute myeloblastic leukemia; AM_0L: acute monocytic leukemia; APL: acute promyelocytic leukemia; ATRA: all-trans retinoic acid; BMMNC: bone marrow mononuclear cells; CML-BC: chronic myelogenous leukemia-blast crisis; HSC: haematopoietic stem cell; IDA: iron-deficiency anemia; MA: megaloblastic anemia; NS: nucleostemin

1 Introduction

Nucleostemin gene first cloned by McKay and Tsai in 2002 was found highly expressed in rat embryo stem cells, rat central nervous system stem cells and rat primitive bone marrow cells. NS gene is apparently involved in regulating the proliferating of both stem cells and at least some types of cancer cell[1]. The protein encoded by NS was abundantly expressed while the cells were proliferating in an early multipotential state, but it abruptly and almost entirely disappeared at the start of differentiation. The fact that NS expressed in stem cells and several cancer cell lines, but not in the differentiated cells of adult tissues, suggested its role in maintaining self-renewal of stem cells and cancer cells[1-3]. Leukemia belongs to malignant clonal disease of haematopoietic stem cell (HSC). Leukemia is characterized by the appearance of increased numbers of immature and dedifferentiation leukemic cells in the marrow and blood. NS is involved in the regulatory pathways, but the fundamentals about NS are still unknown. In the present study, we examined the expression of NS gene in leukemia cell line and patients with acute leukemia

by RT-PCR, which would help illuminate the association of NS gene and leukemia.

2 Materials and Methods

2.1 Cell lines

Two leukemia cell lines, K562 and HL60, were kindly provided by Department of Microbiology and Immunology, School of Medicine, Zhengzhou University and Chinese Academy of Medical Science, respectively. The two cell lines were cultured in RPMI1640 medium (GIBCO-BRI) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin at 37℃ in 5% CO_2 and passaged every 2 – 3 days.

2.2 Subjects

The marrow samples of 39 patients (23 female and 16 male; age 15 – 51 years old, median age 33 years old) with acute leukemia and 11 control subjects were obtained from the First Affiliated Hospital of Zhengzhou University. Among them, patients with M1, M2a, M3, M5a, M5b of AML and ALL were 3, 10, 8, 5, 6 and 7 cases, respectively. Eleven control cases included megaloblastic anemia (MA), iron-deficiency anemia (IDA), the secondary anemia and healthy subjects. The diagnosis standards for acute leukemia were based on the WHO classification of malignant haematological diseases[4].

The bone marrow sample was collected from posterior superior iliac crest and mixed thoroughly with EDTA-K2. The cell smear was determined by Wright-Giemsa staining. The blood-EDTA-K2 solution layered over the Ficoll and centrifuged at 2,000 rpm for 20 min. Cells at the interface between the plasma and Ficoll layer were harvested and washed twice with PBS. The mononuclear cells were collected for RNA extraction.

2.3 Reagents

Trizol reagent was purchased from Invitrogen (Carlsbad, California, USA). Reverse transcription kit, PCR amplification kit, primers synthesis and sequencing were obtained from Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. (Shanghai, China). DNA markers were purchased from MBI (Lansing, Michigan, USA).

2.4 RNA preparation and RT-PCR

Total RNA was extracted from K562, HL60 cell lines and cells were separated with Trizol Reagent according to the manufacture protocol and the separated cells were treated with the DNA-free kit to remove residual genomic DNA. The concentration and integrity of the isolated RNA were determined by UV spectrophotometer (0.2 – 0.9 μg/μl) and gel electrophoresis respectively.

A BLAST search in the GenBank database identified the consensus motif (1,833 bp) of three variants (NM014366, NM206825, NM206826) from NS gene. According to the principle of primer designing, we determined specific primers for NS gene: Up-stream primer 5′-AAAGCCATTCGGGTTGGAGT-3 and Down-stream primer 5′-ACCACAGCAGTTTGGCAGCAC-3′. The amplified fragment was 418 bp. β-actin was used as an internal control, and its full lenghth was 315 bp.

Firstly, isolated RNA (5 μg) was reverse transcribed to cDNA in the 20 μl reaction mixture. Secondly, 5 μl cDNA mixture was used for PCR amplification mixture containing 0.2 μM specific primers, 0.04 μM internal control primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 μl 10× buffer and 1 U Taq DNA polymerase in 25 μl reaction solution. The program was accomplished by 5 min at 95 ℃ for initial denaturing, followed by 30 cycles of 95 ℃ for 30 sec, annealing for 30 sec at 56 ℃ and 72 ℃ for 50 sec and a final extension for 5 min at 72 ℃. The amplified product was identified by gel electrophoresis on 1.7% agrose gel. Furthermore, the sequence of NS gene was exactly identical with the access number of GenBank (NM014366, NM206825, NM206826).

2.5 Determine density score of amplified product

The amplified bands were scanned by gel analysis system. Ratio = density score of positive band/density score of β-actin. The higher the ratio, the higher the expression level of NS gene.

2.6 Statistical analysis

Statistical analysis was performed with SPSS10.0 software. Independent-sample T test was for comparison. $P < 0.05$ was considered statistically significant.

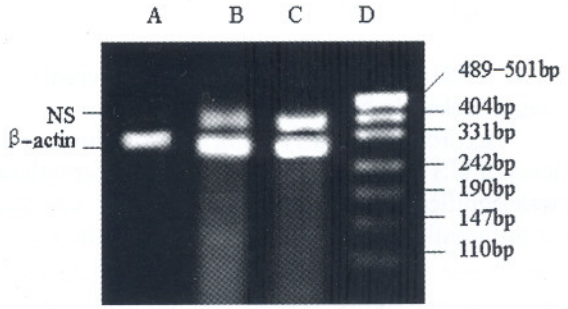

Figure 1. Detection of NS expression in HL60 and K562 cell lines
A: health control; B: HL60; C: K562; D: DNA marker

3 Results

3.1 NS expression in HL60 and K562 cell lines

The expression of NS mRNA in the two cell lines were analyzed by RT-PCR. As shown in Figure 1, there were high levels of NS gene in the two cell lines. Moreover, the expression level of K562 was higher than that of HL60 (0.735 ± 0.22 vs 0.449 ± 0.15, $t = 4.623$, $P < 0.01$).

3.2 NS expression in patients with acute leukemia

The same results were also observed from patients with acute leukemia (Figure 2). The levels of mRNA in different types of leukemia were presented in Table 1.

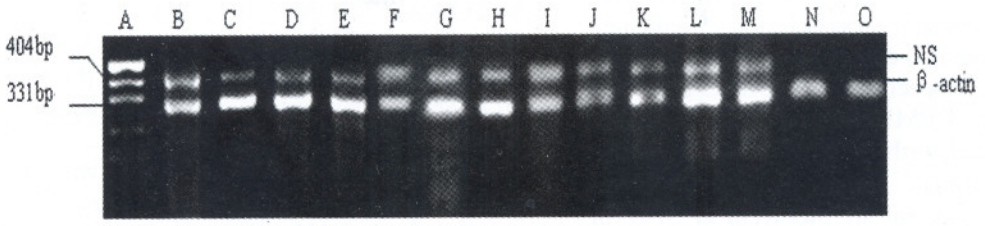

Figure 2. Detection of NS expression in acute leukemia cell
A:DNA marker; B,I,J,L: AML-M1,M2a; C,H,K:ALL; D,G:AML-M3; E,F,M: AML-M5; N:MA control; O:health control

Table 1. Density score of NS expression in patients with different types of leukemia compared with internal control ($\bar{\chi} \pm SD$)

Leukemia	M1 + M2a	M3	M5a	M5b	ALL	Control
Cases	13	8	5	6	7	2
Ratio	0.687 ± 0.21	0.408 ± 0.16	0.866 ± 0.27	0.448 ± 0.19	0.403 ± 0.19	0

3.3 NS expression in healthy subjects and benign anemia subjects

According to the results of RT-PCR, low level or even no NS could be detected in benign anemia and healthy subjects (Figure 3). The reason for low level may be due to the existence of minimal HSC in bone marrow.

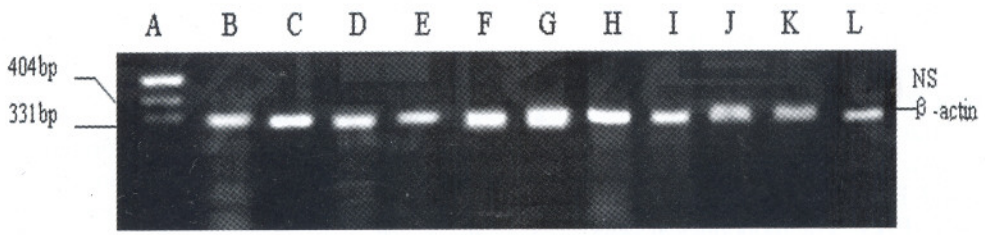

Figure 3. Detection of NS expression in healthy subjects and benign anemia subjects
A:DNA marker; B,E,G,I,L:health subjects; C,H,K:MA; D,F:IDA; J:secondary anemia (chronic inflammations)

3.4 NS expression in different differentiation stages leukemic cells

According to cell types of acute leukemia, the level of NS gene in the early stage of differentiation was significantly higher than that in the late stage of differentiation (Figure 4). That is, the expression levels of NS in K562, M1 + M2a and M5a were higher than in HL60, M3 and M5b, respectively ($t = 4.623$, 3.054, 4.256; $P < 0.01$).

4 Discussion

NS is a newly found p53-binding protein, which exists mainly in the nucleoli of stem cells and various cancer cells, but does not express in committed and terminally differentiated cells[1,5]. The expression level of NS declined obviously during embryo and adult development due to the differentiation of stem cells. *In-vivo* experiments displayed that the NS expression could not even be detected after CNS stem cells were induced to differentiate[2]. In addition, NS expression disappeared before the changes of cell cycle markers during the development of CNS, which indicated the disappearance of NS expression inducing the cell cycle arrest, but not the reverse[1]. Down-regulation of expression of NS gene may result in cell cycle arrest and

cell differentiation[6,7]. Also, NS protein was considered as one of makers of stem cells[8].

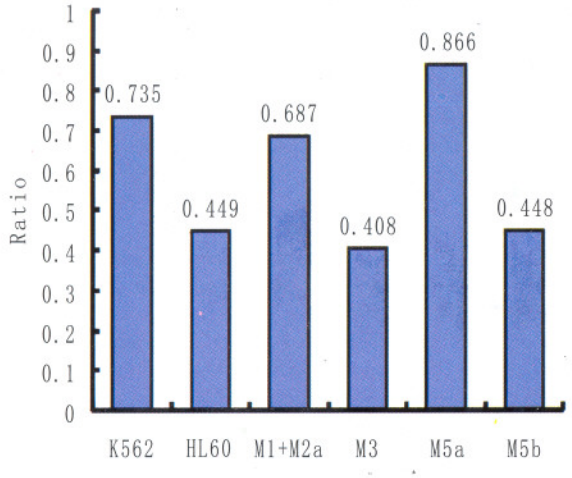

Figure 4. Ratio of density score of NS expression in different differentiation stage of leukemia

Acute leukemia is a group of malignant haematopoietic disorders. Researchers have suspected that similar mechanisms might be at work in both cancer cells and stem cells[6,7]. For example, both have the ability not only to indefinitely proliferate, but also to self-renew. It's the same for leukemia cell. Only one difference is that the indefinite proliferation leukemic cells are maintained in certain stage and do not differentiate and mature. As has been recently proposed, leukemia stem cell may locate on initiative stage of haematopoietic cell clone, with self-renewal capacity, and differentiation arrested before cell mature, which are source of the pathogenesis, drug resistance and relapse of leukemia[9]. Evidence shows that many signals pathways that are classically associated with cancer cells may also regulate normal stem cells self-renew[10]. The preliminary studies indicate that protein encoded by NS gene is involved in regulating the proliferation and differentiation of both stem cells and some types of cancer cells.

In the present study, high levels of NS gene expression were found in leukemic cells. This suggested that NS may play an important role in the origination and development of leukemia. We hypothesized that leukemic cells may lead to cell cycle endlessly rather than cellular differentiation due to the existence of a mass of NS protein. Additionally, we collected healthy subjects and benign anemia subjects as control group. From the results of RT-PCR, very low level of NS was detected in only 2 out of 11 cases of control subjects. This could be because the HSC in bone marrow was very little.

It is noteworthy that there were striking differences in NS level of leukemic cells between different stages of differentiation, such as the expression of NS in K562, M1 and M2a, and M5a was higher than that in HL60, M3 and M5b, respectively. According to previous classifications from immunophenotype, morphology and cell enzymology, differentiation stage of K562 is prior to that of HL60. It is close to the differentiation stage of HSC. Because M1, M2a, and M3 belong to myeloblastic cell type and promyelocytic cell type respectively, differentiation stage of the former is earlier than that of the latter. The same is true in M5a and M5b. In conclusion, there is close relationship between NS expression level and proliferation and dedifferentiation of leukemic cell. However, further study is necessary to verify this point.

K562 is a human erythroleukemia cell line derived from a patient with CML-BC. These cells are pluripotent in that they are able to differentiate along the lineage of granulocytic, megakaryocytic, erythroid, monocytic[11,12]. HL60 cell line is derived from human APL. This pluripotent cell line can differentiate into granulocytic and monocytic cell lineages. Environmental conditions such as pH and many chemical inducers can greatly facilitate the differentiation of HL60 cell line into granulocytic and monocytic cell lineages. For example, ATRA has been reported to induce it granulocytic differentiation, and vitamin D can promote it to differentiate into monocytic series[13]. Any way, there are many similarities between leukemic cell and stem cell. This also further confirms the theory of LSC. However, it remains to be determined how inducers act on the expression of NS gene.

In conclusion, NS gene over-expresses in acute leukemia cells, and it closely relates to the origination and development of leukemia. On the one hand, NS plays an important rcle on regulation of the proliferation and differentiation of leukemia cell. Different differentiation stages of leukemic cell showed different expression levels of NS. On the other hand, low level or no expression could be detected in benign anemia subjects and healthy subjects.

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Tumor-targeting of Bifidobacterium Infantis on Melanoma in Mice

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Abstract: Objective. To investigate the tumor-targeting of Bifidobacterium infantis to melanoma. **Methods.** After bolus administration of Bifidobacterium infantis with 3H-TdR, the values of radioactivity in tumor and organs were examined at 24 h, 48 h, 72 h, 96 h and 168 h. Anaerobic culture and histological observation of tumor and normal organs were taken for the examination of tumor-targeting characteristics of Bifidobacterium infantis. **Results.** The radioactivity in melanoma tissue increased progressively, while the radioactivity in normal organs decreased with time. The anaerobic culture showed an obvious proliferation of Bifidobacterium infantis in tumor tissue. A large part of area was Gram positive in tumor section, whereas the normal tissue was Gram negative. **Conclusion.** Bifidobacterium infantis has good tumor-targeting characteristics in mice melanoma. [Life Science Journal. 2006;3(2):17-20] (ISSN: 1097-8135).

Keywords: Bifidobacterium infantis; melanoma; targeting

1 Introduction

A central problem of gene therapy for cancer is the lack of specificity of current delivering system. It has been proved that a hypoxic region exists in kinds of tumors, especially in solid tumors, and the anaerobic bacteria tend to colonize in a low oxygen environment. Therefore, based on the presence of a hypoxic metabolic region in a solid tumor as well as the tendency of anaerobic bacteria to hypoxic environment, anaerobic bacteria is a potential vector for tumor targeting gene therapy[1-3]. Bifidobacterium infantis is a non-pathogenic anaerobic bacterium. It resides in the intestine of human or rodent animals, which is good for the health of its host. We try to explore the targeting of Bifidobacterium infantis to tumors in the study.

2 Materials and Methods

2.1 Animals

Female C57BL/6 mice aged 6 to 8 weeks were selected from Sichuan University Tumor-research Center.

2.2 Tumor cells preparation

B16-F10 melanoma cells were maintained as monolayer cultures in Dulbecco's medium supplemented with 10% fetal bovine serum. A total of 5×10^5 tumor cells were inoculated into the right thigh muscle of these mice. The solid tumors for study were obtained 2 weeks after inoculation.

2.3 Bacteria culture

Bifidobacterium infantis were cultured under anaerobic condition at 37 ℃ in MRS liquid culture medium. After 24 - 48 h, the concentration was quantified. The original Bifidobacterium infantis suspensions was diluted to $2.5 \times 10^7 - 3.0 \times 10^7$ bacilli/ml with phosphate-buffered saline (PBS) (pH7.4). Finally, Bifidobacterium infantis was injected into four mice from the tail vein of the animals (5-6 million bacilli per mouse) and 2 normal mice were as control.

2.4 Tissue homogenate and culture

After 168 h, six mice were killed. Normal tissue samples were obtained from lung, liver, spleen, kidney and heart. Normal tissue and whole

tumors were excised and minced thoroughly. Each sample was weighted and placed in a homogenizer to prepare a 10% homogenate with cold PBS under aseptic conditions. The diluted tissue homogenates (100 μl/dish) were inoculated into the culture medium (1.5% Briggs agar) respectively. After the agar medium was solidified, all dishes were placed in a completely airtight desiccator. The dishes were cultured at 37 ℃ under anaerobic condition for 3 days.

2.5 Histology

The mice that developed tumors were sacrificed at 168 h after injected with Bifidobacterium infantis. Tumors and normal tissues were excised, fixed in 10% formalin solution, sectioned in paraffin and stained with Gram stain.

2.6 3H-TdR tag

Bifidobacterium infantis were cultured under mixed atmosphere condition (80% N_2, 10% H_2, 10% CO_2). 3H-TdR was added to the bacteria medium(0.37×10^{10} Bq ^{3}H-TdR/10^6 bacilli) after 24 h. Another 12 h later, the bacteria suspension was diluted with cold PBS (pH 7.4), and injected into mice from the tail vein (5 – 6 million bacilli per mouse). The mice were sacrificed at 24 h, 48 h, 72 h, 96 h and 168 h after injection of 3H-TdR tagged Bifidobacterium infantis. Tumors and normal tissues were excised. Finally, the values of radioactivity in each tissue were examined.

3 Results

3.1 The values of radioactivity in tissues

Table 1 showed the radioactivity in various tissues at 24 h, 48 h, 72 h, 96 h and 168 h after intravenous administration. The radioactivity in tumors increased progressively. In contrast, the radioactivity in normal tissues, such as the liver, spleen, kidney and lung were attenuated with time. The values of radioactivity in tissues showed the 3H-TdR tagged Bifidobacterium infantis clustered in tumor tissue, while decreased in normal tissue gradually.

Table 1. The values of tissues' radioactivity (A/g)

Tissue	24 h	48 h	72 h	96 h	168 h
Liver	813	614	568	498	356
Heart	946	1003	823	725	677
Spleen	963	876	625	592	554
Lung	775	776	564	540	434
Kidney	696	505	405	374	356
Tumor	346	370	441	545	778

Each value represents the mean of radioactivity of per gram of tissue.

3.2 Tissue culture

Bacteria colonies were only observed on the agar culture dish inoculating tumor tissue homogenates, but no colonies were observed in any plates inoculating normal tissues (Figure 1 A, B). The anaerobic culture showed an obvious proliferation of Bifidobacterium infantis in tumor tissue.

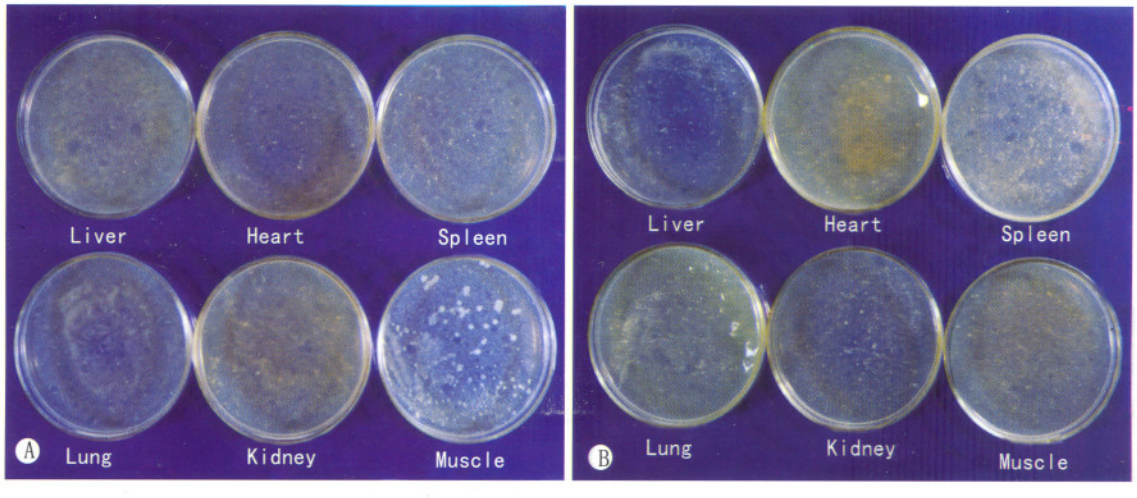

Figure 1. Comparision of the anaerobic culture results of bacilli in tissues after seven days of Bifidobacterium infantis administration
A: Tumor and normal tissues from tumor-bearing mice; B: Normal tissues from normal mice

3.3 Histology

Four mice that developed melanoma tumors were injected intravenously with Bifidobacterium infantis, killed 168 h later and examined for the presence of Gram-positive Bifidobacterial rods in both tumors and normal tissues. Figure 2 A, B showed numerous bacilli were scattered or clustered either on the border between the necrotic and non-necrotic regions or in the necrotic region of tumors. In contrast, no evidence of bacteria was observed in the normal tissue segment.

4 Discussion

A crucial difficulty for cancer gene therapy is the lack of specificity of current delivery systems. After i.v. inoculation of Bifidobacterium infantis to tumor-bearing mice, we initially observed a distribution of viable bacilli throughout the body, but after 96 – 168 h, most of bacilli were accumulated in the tumor tissue. In this report, we demonstrated the results with the methods of 3H-TdR tagged and the values of radioactivity in tumor and normal organs. The fact that the bacilli can colonize and proliferate in the tumor tissue implies that this tissue possesses an environment that is suitable for the growth of this bacterium. Vaupel[4] made his study on cancer patients using oxygen electrode measurement. In his study, Vaupel found the average oxygen partial pressure in normal tissues read 24 – 66 mmHg, whereas the readings dropped to 10 – 30 mmHg in a tumor tissue with a marked central region where the readings went below 2.5 mmHg. It is evident that the center of solid tumor is generally at low level of oxygen, and anaerobic bacteria tend to colonize in a low oxygen environment[5].

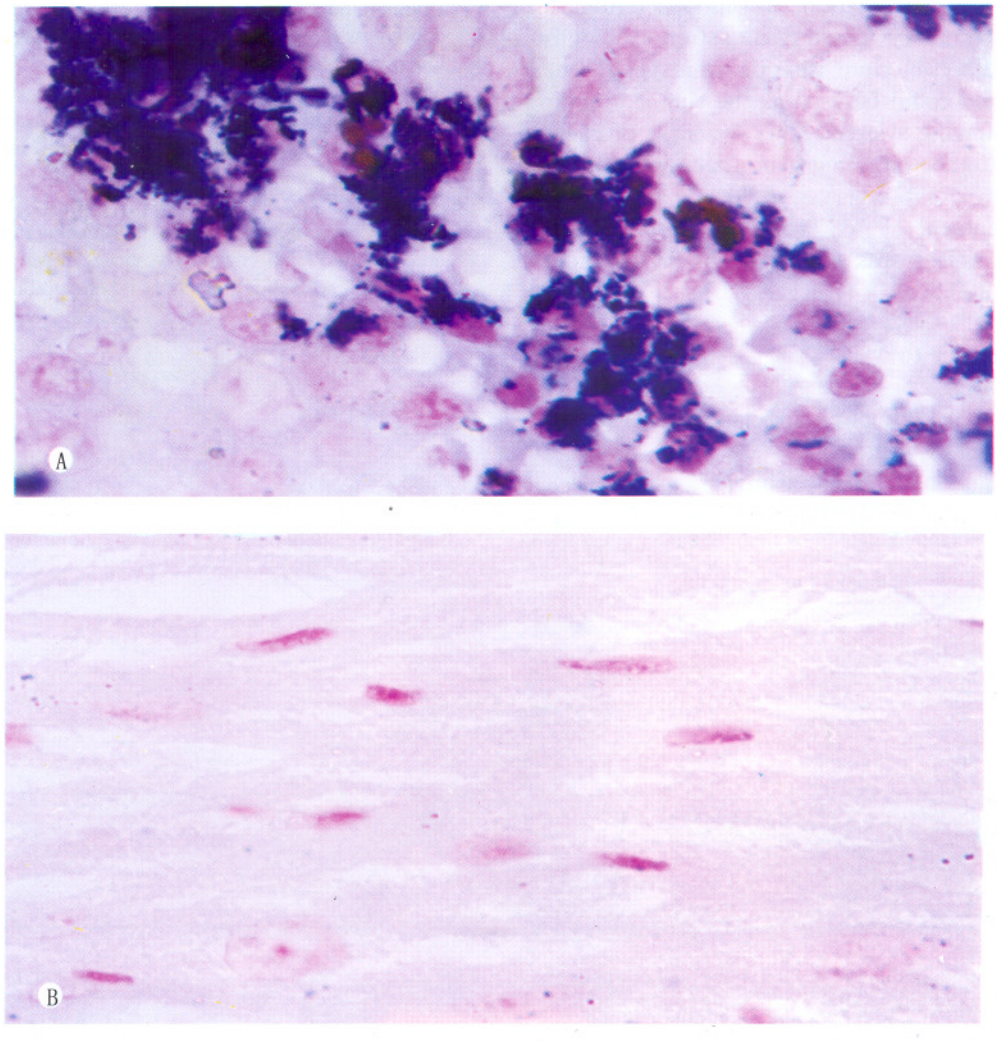

Figure 2. Photomicrograph of sections stained by the Gram method (×100)
A: Melanoma tumor tissue; B: Heart tissue

Bifidobacterium infantis are Gram-positive, domestic, non-pathogenic bacteria, found in the lower small and large intestine of humans and other animals. In addition, these bacteria have health-promoting properties for their hosts by, for example, increasing the immune response[6], inhibiting carcinogenesis[2,7] and protecting the host against viral infection[8,9]. The non-pathogenesis and importance of these microorganisms are now generally acknowledged. Based on the presence of a hypoxic metabolic region in a solid tumor and tendency of anaerobic bacteria to hypoxic environment, Bifidobacterium infantis can be used as a transfer vectors for tumor targeting gene therapy.

In this study, the anaerobic culture showed an obvious proliferation of Bifidobacterium infantis in tumor tissue. A large part of area was Gram-positive in the tumor tissue section, whereas the normal tissue was Gram-negative. We demonstrated the tumor-specific germination of Bifidobacterium infantis. In summary, these results strongly suggested that Bifidobacterium infantis were good tumor targeting and could be used as the tumor targeting gene-transferring system. This system is promising as a novel tumor targeting gene therapy system.

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Expression of HspA-UreB Fusion Protein of *Helicobacter pylori* and Its Immunocompetence

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Abstract: Objective. To construct recombinant plasmid expressing HspA-UreB fusion protein of *H pylori*, and to determine its immunocompetence. **Methods.** The *hspA* and *ureB* genes were amplified by PCR from *H pylori* strain MEL-HP27 isolated in Zhengzhou and cloned directly into vector pET30a, and the recombinant plasmid was then transformed into *E. coli* BL21DE3. The recombinant plasmid was induced to express fusion protein HspA-UreB in *E. coli* by isopropylthio-β-D-galactoside (IPTG). The protein was analyzed by SDS-PAGE, purified by Ni^{2+} affinity chromatography, and immunized the mice. The immunoreactivity of the fusion protein were analyzed by Western blot. **Results.** In comparison with the reported corresponding sequence from Genbank, the nucleotide sequence homologies of the cloned *hspA* and *ureB* genes were 95.20 – 97.48% and 96.08 – 98.30%, and their putative amino acid sequence homologies were 95.76 – 97.46% and 98.77 – 99.82% for the two genes, respectively. The results of SDS-PAGE and optical density scanning indicated that the fusion protein was expressed by *pET30a-hspA-ureB*-BL21DE3 as a protein with *Mr* 82,100 of molecular weight and was 21% of the total bacterial proteins. The purity of fusion protein was 91%, and could be recognized by the serum from *H pylori* infected patients and mice immunized with purified HspA-UreB fusion protein. **Conclusion.** A recombinant plasmid expressing fusion protein HspA-UreB of *H pylori* was constructed and identified with good immunocompetence, and it suggested that HspA-UreB might be a potential vaccine antigen for controlling and treating *H pylori* infection. [Life Science Journal. 2006;3(2):21 – 26] (ISSN: 1097 – 8135).

Keywords: *Helicobacter pylori*; HspA-UreB; immunocompetence

Abbreviations: HapA: *Helicobacter pylori* adhesion A; HspA: heat shock protein subunit A; Lpp20: lipoprotein 20; NAP: neutrophil-activating protein; Omp: outer membrane protein; UreB: urease subunit B; VacA: vacoulating cytotoxin A

1 Introduction

Helicobacter pylori (*H pylori*) infection is a major cause of chronic active gastritis and most peptic ulcer diseases[1-3], and also closely related to gastric cancers[4,5]. This microorganism has been categorized as class I carcinogen by the World Health Organization[6]. For this reason, successful eradication of *H pylori* may be an important goal for this study. Currently, the treatment for *H pylori* infection involves antibiotic therapy, but this has some disadvantages such as increasing the expense and strains resistance[7,8]. An alternative approach is to develop a vaccine, which could eradicate *H pylori* infection[9,10]. Selection of antigenic epitope is critical in developing *H pylori* vaccine. The majority of studies attempting to produce a vaccine have focused on urease enzyme[11,12], heat shock protein[13,14] and vacuolating cytotoxin[15]. The protection afforded by a single antigen is not enough[16], but the two kinds antigen HspA and UreB combined could provide 100% protection for mice from being infected with *H pylori*.

In this study, the recombinant expression system for HspA-UreB fusion protein of *H pylori* was constructed. Immunoreactivity and immunogenicity of HspA-UreB fusion protein were further examined. The results of this study may contribute to the development of *H pylori* vaccines.

2 Materials and Methods

2.1 Materials

A clinical strain of *H pylori*, MEL-HP27 was isolated from a patient with chronic gastritis. Bacterial strain BL21 (DE3) and plasmid pET-30a were purchased from Novagen (Madison WI, USA). Primers for PCR amplification and restriction endonucleases *Sal* I, *Xho* I, *EcoR* I and T4

DNA ligase were purchased from Sangon (Shanghai, China). The Pyrobest™ high fidelity DNA polymerase and isopropyl-β-D-thiogalatopyranoside (IPTG) were purchased from Takara Company (Dalian, Jilin, China). Goat anti-mouse and goat anti-human IgG-HRP were purchased from Bangding Bioengineering company (Beijing, China). The mice were provided by the Center of Experimental Animal of Henan (Zhengzhou, Henan, China).

2.2 Construction of expression system *pET30a-hspA-ureB*-BL21 (DE3)

Genomic DNA of MEL-HP27 was extracted by conventional phenol-chloroform method. Oligonucleotide primers were designed based on the corresponding genomic sequence of international standard strain NCTC11637. The sequence of *hspA* sense primer with an endonuclease site of EcoR I was 5′-CCC GAA TTC ATG AAG TTT CAA CCA TTA-3′. The sequence of *hspA* antisense primer with an endonuclease site of *Sal* I was 5′-CGC GTC GAC GTG TTT TTT GTG ATC ATG AC- 3′. The sequence of *ureB* sense primer with an endonuclease site of *Sal* I was 5′- CC GTC GAC AAA AAG ATT AGC AGA AAA G-3′. The sequence of *ureB* antisense primer with an endonuclease site of *Xho* I was 5′- CGC CTC GAG CTA GAA AAT GCT AAA GAG-3′. PCR was performed with the hot start method.

The parameters for PCR were at 95 ℃ for 5 min, ×1; at 94 ℃ for 1 min, at 55 ℃ for *hspA* gene for 1 min (for 3 min for ureB gene), at 72 ℃ for 1 min, ×30; then at 72 ℃ for 10 min, ×1. The results of PCR were observed under UV light after electrophoresis. The expected sizes of target amplification fragments were 354 bp for *hspA* gene and 2710 bp for *ureB* gene.

PCR products of *hspA* gene digested with *EcoR* I and *Sal* I, and *ureB* gene digested with *Sal* I and *Xho* I, then were inserted into *EcoR* I and *Xho* I restriction fragments of the expression vector *pET30a* using T4 DNA ligase. The recombinant expression plasmids *pET30a-hspA-ureB* were transformed into competent *E. coli* BL21 (DE3), and the expression systems were named as *pET30a-hspA-ureB*-BL21 (DE3). The target fragments of *hspA* and *ureB* genes inserted in *pET30a* plasmid were sequenced by Sangon Company (China).

2.3 Expression, purification and identification of fusion proteins

The recombinant strains were incubated overnight at 37℃ while shaking in 5 ml LB medium with 100 μg/mL kanamycin, and the cell grew until the optical density at 600 nm reached 0.4 – 0.6. IPTG was added to a final concentration of 0.3 mmol/L. The cells growing for 4 h after induction were harvested by centrifugation at 4,000 g for 20 min. The molecular weight and output of HspA-UreB fusion protein were examined by SDS-PAGE. The serum of patient infected with *H pylori* and commercial goat-human HRP-IgG were used as the first and second antibodies to identify the immnuoreactivity of HspA-UreB. The recombinant *E. coli* cells growing in 50 ml LB medium with kanamycine for 3 h induced by IPTG harvested by centrifugation at 4,000 g for 20 min. The bacterial pellet resuspended in pure water was ultrasonically broken (300v, 4s×20), centrifugated at 12,000 g for 15 min. The recombinant HspA-UreB fusion protein was collected by Ni-NTA affinity chromatography and analyzed by electrophoresis in a 10% polyacrylamide gel.

2.4 Immunization of mice

Six to eight weeks old mice were immunized five times by hypodermic injection in the back at weekly intervals. Each dose consisted of 5 μg adjuvant. One week after the last immunization blood samples were taken from eyes. The sera were separated, and stored at −20 ℃ until assay.

2.5 Serum antibody response

The recombinant HspA-UreB fusion protein was electrophoresed as in SDS-PAGE and then were transformed to PVDF membrane. The mice sera immunized with HspA-UreB and goat anti-mouse HRD-IgG were used as the first and second antibodies to perform Western blot.

3 Results

3.1 PCR amplification of *H pylori hspA* and *ureB* genes

Target fragments of *hspA* and *ureB* genes with expected sizes amplified from DNA template of *H pylori* MEL-HP27 were shown in Figure 1.

3.2 Identification of recombinant plasmid

After extracting plasmids DNA from recombinant *E. coli* strains, the recombinant plasmids were digested by *EcoR* I or *Xho* I, and by *EcoR* I and *Xho* I simultaneously, then the digestive products were visualized on 10 g/L agarose gel eletrophorese (Figure 2). It demonstrated that recombinant plasmid contained the objective fusion gene.

3.3 Nucleotide sequence analysis

The nucleotide sequences of *hspA* and *ureB* gene in *pET30a-hspA-ureB* were listed in Figure 3 and Figure 4. The homologies of nucleotide and putative amino acid sequences of the cloned *hspA* gene compared with the published *hspA* sequences were

from 95.20% to 97.48% and from 95.20% to 97.46%, respectively (Table 1). The homologies of nucleotide and putative amino acid sequences of the cloned *uerB* gene were from 96.08% to 98.30% and from 98.77% to 99.65 % (Table 2).

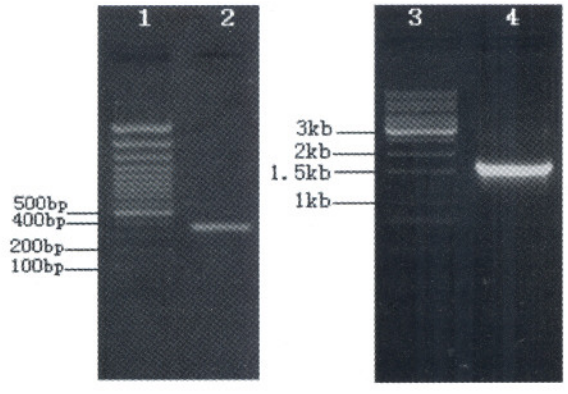

Figure 1. Target fragments of *hspA* and *ureB* genes amplified from *H pylori* strain MEL-HP27
Lane 1: 100 bp DNA ladder marker; Lane 2: PCR products of *hspA* gene; Lane 3: 1 kb DNA ladder marker; Lane 4: PCR products of *ureB* gene.

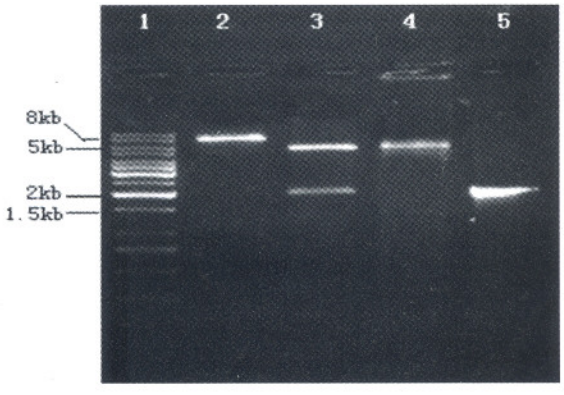

Figure 2. Identification of recombinant plasmid by restriction enzyme digestion
Lane 1: 1kb DNA ladder marker; Lane 2: recombinant plasmid digested by *EcoR* I; Lane 3: recombinant plasmid digested by *EcoR* I and *Xho* I; Lane 4: *pET30a* digested by *EcoR* I; Lane 5: products of *hspA-ureB* fusion gene from recombinant plasmid.

Table 1. Homology comparison of *H pylori hspA* gene sequences

H pylori strains compared with MEL-HP27	Different base pair	Homology of nucleotide	Different amoni acid	Homology of amoni acid
NCTC11637	9	97.48%	3	97.46%
26695	11	96.89%	3	97.46%
CH-CTX1	17	95.20%	5	95.76%
J99	10	97.18%	4	96.61%

Table 2. Homology comparison of *H pylori ureB* gene sequences

H pylori strains compared with MEL-HP27	Different base pair	Homology of nucleotide	Different amoni acid	Homology of amoni acid
NCTC11637	40	97.67%	3	99.47%
HPK5	29	98.30%	1	98.82%
26695	46	97.31%	6	98.95%
HP031	49	97.13%	5	99.12%
J99	67	96.08%	2	99.65%
CH-CTX1	51	97.01%	7	98.77%

3.4 Expression, purification and identification of HspA-UreB fusion protein

IPTG at concentration of 0.3 mmol/L efficiently induced the expression of HspA-UreB in the *pET30a-hspA-ureB*-BL21DE3 system. SDS-PAGE analysis showed that the clearly identifiable band with *Mr* 82,100 highly expressed fusion proteins, which was similar to that predicted.

The output of HspA-UreB fusion protein was approximate 21% of the total bacterial proteins (Figure 5). Among them, soluble substance accounted for 30% of supernatant. HspA-UreB fusion protein was further purified with Ni-NTA column, its final purity was 91%. Western blot also showed an identifiable blot band with *Mr* 82,100.

3.5 Antigenicity of recombinant HspA-UreB fusion protein

An immunized reacting band with the weight of *Mr* 82,100 appeared in the Western blot (Figure 6) in which the purified HspA-UreB fusion protein reacted against the serum of mouse immunized by HspA-UreB.

1 atgaagtttc taccattagg agaaagggtc ttagtagaaa gacttgaaga agagaacaaa
61 accagttcag gcatcatcat ccctgataac gctaaagaaa agcctttaat gggcgtagtc
121 aaagcggtta gccataaaat cagcgagggt tgcaaatgcg ttaaagaagg cgatgtgatc
181 gcttttggca aatacaaagg cgcagaaatc gttttagacg gcgttgaata catggtgcta
241 gagctagaag acattctagg tattgtgggc tcaggctctt gttgtcatac aaatagtcat
301 gaccataaac atgctaaaga gcatgaagct tgctgtcatg atcacaaaaa acactaa

Figure 3. *hspA* nucleotide sequence of *H pylori* strain MEL-HP27

1 atgaaaaaga ttagcagaaa agaatatgtt tctatgtatg gccctactac aggcgataaa
61 gtgagattgg gcgatacaga cttgatcgct gaagtagaac atgactacac catttatggc
121 gaagagctta aattcggtgg cggtaaaact ttgagagaag gcatgagcca atccaacaac
181 cctagcaaag aagaactgga tttaatcatc actaacgctt taatcgtgga ttacaccggt
241 atttataaag cggatattgg tattaaagat ggcaaaatcg ctggcattgg caaaggcggc
301 aacaaagaca tgcaagatgg cgttaaaaac aatcttagcg tgggtcctgc tactgaagcc
361 ttagctggtg aaggtttgat cgtaactgct ggtggtattg acacacacat ccacttcatc
421 tccccccaac aaatccctac agcttttgca agcggtgtaa caacgatgat tggtggcgga
481 actggccctg ctgatggcac taacgcaacc actatcactc caggcagaag aaatttaaaa
541 tggatgctca gagcggctga agaatattct atgaatttag gtttcttagc taaaggtaac
601 gcttctaatg atgcgagctt agccgatcaa attgaagccg gtgcgattgg ctttaaaatc
661 catgaagact ggggaacaac tccttctgca atcaatcatg cgttagatgt tgcggacaaa
721 tacgatgtgc aagtcgctat ccatacggac actttgaatg aagccggttg tgtagaagac
781 actatggcag ccattgccgg acgcactatg cacactttcc acactgaagg cgctggtggc
841 ggacacgctc ctgatatcat taaagtagcc ggcgaacaca acattctgcc cgcttccact
901 aaccccacta tcccttтcac tgtgaataca gaagcagaac acatggacat gcttatggtg
961 tgccaccact tggataaaag cattaaagaa gatgttcagt tcgctgattc aaggatccgc
1021 cctcaaacca ttgcggctga agacactttg catgacatgg ggattttctc aatcactagt
1081 tctgactctc aagctatggg tcgtgtgggt gaagttatca ccagaacttg gcaaacagct
1141 gacaaaaaca aaaaagaatt tggccgcttg aaagaagaaa aaggcgataa cgacaacttc
1201 agaatcaaac gctacttgtc taaatacacc attaacccag cgatcgctca tgggattagc
1261 gagtatgtag gttctgtaga agtgggcaaa gtggctgact tggtattgtg gagtccagca
1321 ttctttggcg tgaaacccaa catgatcatc aaaggtgggt ttattgcatt gagtcaaatg
1381 ggcgatgcga acgcttctat ccctacccca caaccagttt attacagaga aatgttcgct
1441 catcatggta aagccaaata cgatgcaaac atcacttttg tgtctaaagc ggcttatgac
1501 aaaggcatta aagaagaatt agggcttgaa agacaagtgt tgccggtaaa aaattgcaga
1561 aacatcacta aaaaagacat gcaattcaac gacactaccg ctcacattga agtcaatcct
1621 gaaacttacc atgtgttcgt ggatggcaaa gaagtaactt ctaaaccagc cactaaagtg
1681 agcttggcgc aactctttag cattttctag

Figure 4. *ureB* nucleotide sequence of *H pylori* strain MEL-HP27

4 Discussion

Immune protection function of several *H pylori* antigens has been studied and investigated, such as UreB[17,18], HspA[19], VacA[15], Catalase[20,21]. Several studies demonstrated that three of *H pylori* antigens, such as Lpp20[22,23], NAP[24,25], HpaA[26], Omp[27] are also excellent and ideal antigens that can be potentially used for the development of *H pylori* vaccine. However, some researches indicated that immune protection function of combined antigens was better than single antigen. Recently, some researchers study poly-antigen genetic engineering vaccine of *H pylori* in China[27,28]. This research selected two kinds of antigens with effective immunization: HspA and UreB, to construct the expression system for *hspA-ureB* fusion gene, and to determine the immunogenicity and immunoreactivity of HspA-UreB fusion protein. UreB, used as a candidate antigen for *H pylori* genetic engineering vaccine, has advantages of high sequence conservation, high frequency of distribution, large expression in different isolates, strong antigenicity due to its big molecular mass, and granular structure and exposure on the

surface of bacteria[29,30]. HspA is another candidate antigen for *H pylori* vaccine, which is termed as "molecular chaperone" because they assist in post-translational assembly, secretion, and stability of oligomeric protein structures[31].

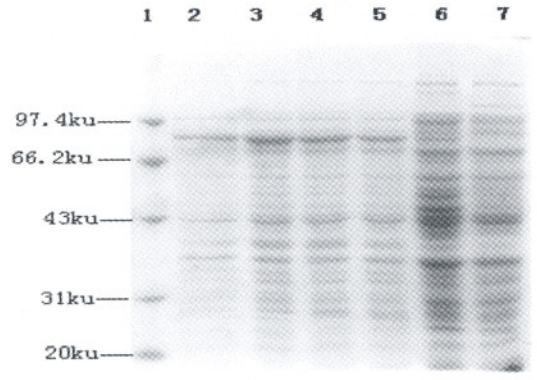

Figure 5. SDS-PAGE analysis of HspA-UreB induced by IPTG at different time
Lane 1: molecular weight marker; Lanes 2-5: BL21 (DE3) with *pET-hspA-ureB* induced for 4, 3, 2 and 1 h; Lane 6: BL21 (DE3) with *pET-hspA-ureB* uninduced for 4 h; Lane 7: BL21 (DE3) with *pET30a* induced for 4 h.

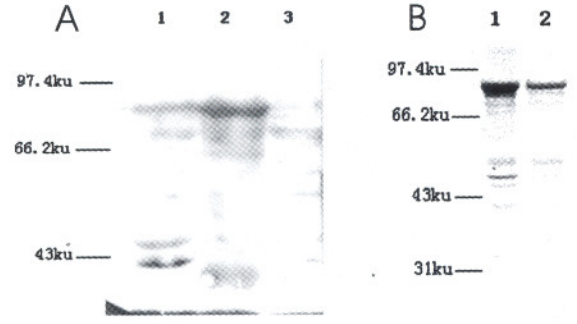

Figure 6. Western blot result of mouse serum against recombinant HspA-UreB
Lane A1: BL21 (DE3) with *pET-hspA-ureB* uninduced for 4 h;
Lane A2: BL21 (DE3) with *pET-hspA-ureB* induced for 4 h;
Lane A3: BL21 (DE3) with *pET30a* induced for 4 h;
Lane B1: unpurified HspA-UreB; Lane B2: purified HspA-UreB.

In this study, the recombinant plasmids expressing HspA-UreB fusion gene of *H pylori* were constructed. The *hspA* gene cloned from *H pylori* strain MEL-HP27 showed high homologies of the nucleotide and putative amino acid sequences compared with five published corresponding sequences (Table 1). Similarly, the homologies of nucleotide and putative amino acid sequences of the clone *ureB* gene from *H pylori* strain MEL-HP27 were quite high when compared with the published corresponding sequences (Table 2).

The results of SDS-PAGE demonstrated that the constructed expression system *pET30a-hspA-ureB*-BL21 (DE3) efficiently produced the target fusion protein. The most output of fusion protein HspA-UreB within whole cell protein was about 21% of the total bacterial proteins, which is beneficial to industrial production.

Western blot assay was performed in this study to confirm that the purified fusion protein HspA-UreB could be recognized by the serum from patient infected with *H pylori* and also be recognized by the sera from mice immunized with purified fusion protein. HspA-UreB exhibited favorable immunogenicity and immunoreactivity.

In conclusion, HspA-UreB is excellent and ideal candidate antigen that can be potentially used for the development of *H pylori* vaccine.

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Detection of MAGE-A3 Antigen and HLA-class I Genes Distribution in Lung Cancer

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Abstract: Aim. In order to speculate the ratio of lung cancer patients who can be treated with immunotherapy based on MAGE-A3 antigen, the expression of MAGE-A3 antigen and the distribution of HLA-class I genes were investigated in lung cancer tissues. **Methods.** SDS-PAGE and Western blot were to detect the expression of MAGE-A3 antigen in 63 lung cancer patients. The distributions of HLA-A1, A2 and A24 gene in 70 lung cancer patients and the tissues adjacent were detected with PCR-SSP. **Results.** 1) 31 of 63 lung cancer tissues, and 5 tumor adjacent tissues expressed MAGE-A3 antigen; 2) In 70 lung cancer patients the distribution of HLA-A1, A2 and A24 were 4.28%, 54.28% and 50.0%, respectively; 3) In 70 tumor adjacent tissues the distribution of HLA-A1, A2, A24 were 4.28%, 60.0% and 52.86%, respectively. The total positive rate of HLA-A1, A2, A24 was 80.0% in lung cancer tissues. **Conclusions.** There were about 39% lung cancer patients who could be treated with immunotherapy based on MAGE-A3 antigen. [Life Science Journal. 2006;3(2):27-31] (ISSN: 1097-8135).

Keywords: lung neoplasm; MAGE-A3; HLA-I molecules; immunotherapy

Abbreviations: CTL: cytotoxic T lymphocyte; HLA: human leukocyte antigen; MAGE: melanoma antigen

1 Introduction

The MAGE-A3 is a member of MAGE family, whose antigen is known to be neo-expressed in a large proportion of tumors but who is not detectable in normal tissues, and who could be a target antigen recognized by autologous cytotoxic T lymphocytes (CTLs). Such tumor cells generating MAGE-A3 antigen could be an ideal target to carry out tumor specific immunotherapy[1]. In addition HLA class I molecule exerts very important effect in the process that MAGE-A3 antigen induces the CTLs proliferation[2]. This experiment detected the expression levels of MAGE-A3 antigen in lung cancer patients. At the same time we quested for distribution of three HLA class I alleles (HLA-A1, A2, A24) in lung cancer patients, which could be combined with MAGE-A3 antigen specifically. The percentage was measured of lung cancer patients who were able to be treated with immunotherapy based on MAGE-A3 antigen.

2 Materials and Methods

2.1 Sample collection

70 fresh surgical specimens were obtained from the First Affiliated Hospital of Zhengzhou University, the Second Affiliated Hospital of Zhengzhou University and Henan Province Chest Hospital, repectively. The samples include: 37 squamous cancers, 22 adenocarcinomas, 5 squamous-adenocarcinomas, 2 big cell lung cancers, 3 small cell lung cancers and 1 hybrid lung cancer were mixed big and small cells. All tissues were frozen in liquid nitrogen immediately after surgery and stored at −80℃ until the extraction of protein.

2.2 Protein extraction

The protein was extracted by SDS-PAGE and stored at −20℃.

2.3 DNA extraction

UNIQ-10 genome DNA extraction kit (Shanghai, China). DNA solution was stored at −20℃.

2.4 Western blot

Protein samples were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked with 1% protein powder (Wuhan BOSTER Ltd., Wuhan, Hubei, China) in PBS for 2 hours at room temperature or overnight at 4 ℃, and incubated with primary antibody − 57B (generously presented by Giulio C. Spagnoli, M. D.) at room temperature for 2 hours; protein bands in membrane were stained with a horseradish peroxidase-conjugated secondary antibody − Goat anti-mouse IgG and counterstained with 3′3-diaminobenzidine (DAB) finally. Pictures were captured by Bio Imaging System (USA).

2.5 PCR with sequence specific primer (PCR-

SSP)

The sequence specific primers of HLA-A1, HLA-A2 and HLA-A24 were designed according to the principle of primer design and their full sequences of primers were showed in Table 1.

Table 1. Sequence specific primers

Gene	Region	Primer sequence	PCR fragment (bp)
HLA-A1	Ex2: 113~130	5′-CGACGCCGCGAGCCAGAA-3′	557
	Ex3:216~232	5′-AGCCCGTCCACGCACCG-3′	
HLA-A2	Ex2: 149~167	5′-GTGGATAGAGCAGGAGGGT-3′	489
	Ex3:110~128	5′-CCAAGAGCGCAGGTCCTCT-3′	
HLA-A24	Ex2: 166~184	5′-GGCCGGAGTATTGGGACGA-3′	629
	Ex3: 195~213	5′- CCTCCAGGTAGGCTCTCTG-3′	

DNA was extracted from lung cancer and cancer adjacent tissues according to the manufacture instructions. DNA purifying and concentrating were measured by spectrophotometry. 100 ng DNA was used for PCR-SSP (total volume: 30 μL). Whatman Biometra Tgradient 96 PCR (Germany) was used to carry out the PCR, with a program of preheating for 5 min at 95 ℃, followed by 5 cycles of 30 sec at 95 ℃, 50 sec at 65 ℃, and 50 sec at 72 ℃; 5 cycles of 30 sec at 95 ℃, 50 sec at 60 ℃, and 50 sec at 72 ℃. Last 20 cycles was carried out of 30 sec at 95 ℃, 50 sec at 57 ℃, and 50 sec at 72 ℃. The final step was 5 min at 72 ℃. All PCR products were analyzed by electrophoresis in agarose with TAE buffer (0.04 M Trisacetate, 0.001 M EDTA, pH 8.0).

2.6 Statistics analysis

Experimental data was analyzed by SPSS software (10.0). Difference between two independent ratios was determined with Chi-square test. Significance was assumed at $P<0.05$. If the number of samples was less than 40 ($n<40$) the rank sum test would be used for analysis.

3 Results

3.1 The relationship of MAGE-A3 antigen and different clinical pathologic types and stages (Table 2, Figure 1)

Table 2. The expression of MAGE-A3 antigen in lung cancer tissues and adjacent tissues

Tissue type	Cancer tissue			Cancer adjacent		
	positive	negative	total	positive	negative	total
SC[a]	15	15	30	3	27	30
GC[b]	10	12	22	1	21	22
GSC[c]	4	1	5	1	4	5
NDC[d]	2	4	6	0	6	6
Total	31	32	63	5	58	63

a: squamous cancer; b: adenocarcinoma; c: squamous-adenocarcinoma; d: non-differential cancer

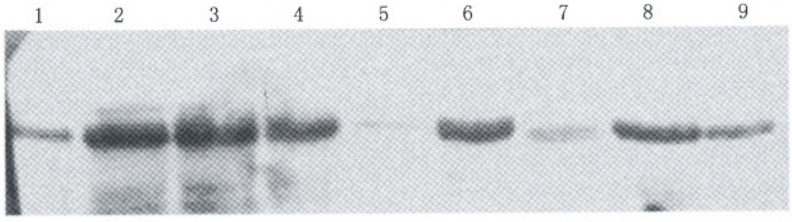

Figure 1. Western blot analysis on the expression of MAGE-A3 antigen in lung cancer patients
Lane 1, 3, 5, 7: cancer adjacent tissues; Lane 2, 4, 6, 8, 9: cancer tissues

Expression of MAGE-A3 antigenic protein: Among 63 samples MAGE-A3 antigen was expressed in 31 cases. The ratio of MAGE-A3 antigen expression was 49.21% in lung cancer. There was no significance between different pathological clinical types and different pathological stages. In addition, 5 samples of tumor adjacent tissues also expressed MAGE-A3 antigen as well as their cancer tissue.

3.2 The results of PCR-SSP (Figures 2, 3, 4)

The distribution of HLA-class I molecules by DNA typing: In tumor tissues the positive rates of

HLA-A1, HLA-A2 and HLA-A24 were 4.28% (3/70), 54.28% (38/70) and 50.0% (35/70), respectively. In para-tumor tissues the positive rates were 4.28% (3/70), 60.0% (42/70) and 52.86% (37/70), respectively. The total positive rate of HLA-A1, A2, A24 was 80.0% (56/70) in lung cancer patients.

3.3 The relationship of HLA-A1, A2, A24 and different clinical pathological types and stages of lung cancer (Tables 3, 4, 5)

There were no significant differences of lung cancer tissues and cancer adjacent tissues of these three alleles ($P>0.05$). In addition no significant differences in different pathological types and different pathological stages of lung cancer were found. The positively expressed MAGE-3 antigen and HLA-class I (A1, A2, A24) molecules were about 39.36% (80.0% × 49.21%) in lung cancer patients.

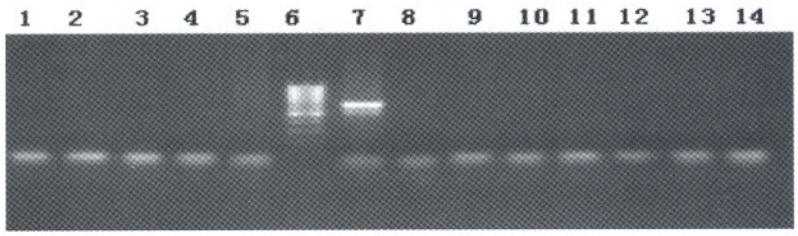

Figure 2. The result of HLA-A1 PCR-SSP in lung cancer patients

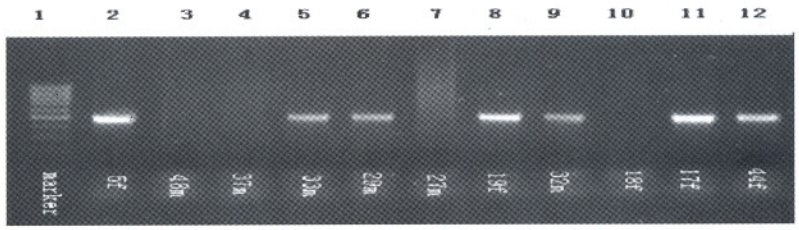

Figure 3. The result of HLA-A2 PCR-SSP in lung cancer patients

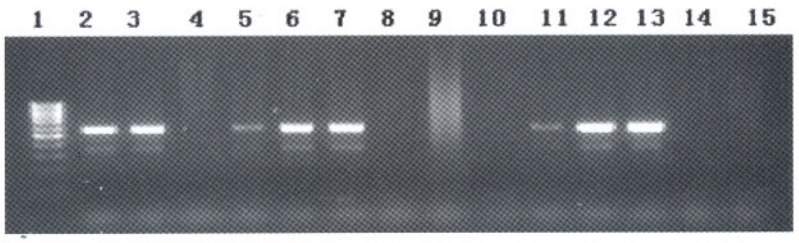

Figure 4. The result of HLA-A24 PCR-SSP in lung cancer patients

4 Discussion

4.1 The expression of MAGE-A3 antigen in lung cancer

The human MAGE-A3 gene family consists of a large number of chromosome-X-linked genes originally identified because they encode the products that can be recognized by autologous cytotoxic T cells. For the MAGE genes don't express in normal adult tissues except testis while express in a large variety of neoplastic lesions, so they are considered as tumor-specific antigens and ideal targets for cancer immunotherapy. Numerous CTL epitopes from the MAGE-A3 antigen have been identified, which were found to be restricted by commonly found MHC class I alleles such as HLA-A1, HLA-A2, HLA-A24, HLA-B37 and HLA-B44. As a tumor specific antigen it can act with a lot of immune cells such as antigen process cells (APCs) and autologous cytotoxic T lymphocyte etc. The interaction between various cells determined the fate of tumor cell at a large extent.

Currently, there are a lot of reports about the expression of MAGE-A3 in tumor tissues at home and abroad, but much concentrated on its mRNA level[3], few on its protein level and the result dis-

agreement with each other. In this experiment we found that the positive expression of MAGE-A3 antigen was 49.21%, higher than Liu's report[4], in accordance with Bolli's report[5]. The result that there was no significant difference in different pathological types and different pathological stages of lung cancer was in accordance with the result of most reports, which indicated that the expression of MAGE-A3 antigen was an independent index in above-mentioned clinical pathological characteristic. Different pathological types and different pathological stages of lung cancer patient can't affect the tumor specific immune therapy based on MAGE-A3 antigen. We found that the MAGE genes expressed in not only lung cancer tissues but also some normal lung tissues adjacent to cancers (16.13%, 5/31). And by pathological examination these normal lung tissues appeared hyperplasia of fibrous tissues and epithelial tissues (namely heterogeneous histiocytes), which suggested that the activation of MAGE genes could occur at a very early stage of lung carcinogenesis. This result was in accordance with a few reports[6,7] but opposite to most reports. It may be because some of cancer adjacent tissues have already occurred malignant conversion in the early stage in which we couldn't find any typical cancer cells by pathological examination. Furthermore, it showed that MAGE-A3 antigen gene may have already been activated before malignant conversion of normal cells. Some articles reported that MAGE-A3 gene's activation, transcription and expression were determined by the promoter's degree of methylation[8]. The carcinogen may activate MAGE-A3 gene through changing its promoter's methylation. In our opinion, the activation of MAGE was a common phenomenon in carcinogen-exposed lung tissue as well as in lung cancer tissue. Further work is needed about the expression of MAGE-A3 in lung cancer adjacent tissues.

Table 3. The distribution of HLA-A1, A2, A24 in lung cancer and cancer adjacent (n)

Genotype	Cancer			Cancer adjacent		
	Positive	Negative	Total	Positive	Negative	Total
HLA-A1	3	67	70	3	67	70
HLA-A2	38	32	70	42	28	70
HLA-A24	35	35	70	37	33	70

Table 4. The distribution of HLA- A2 in different pathological types of lung cancer (n)

Pathologic type	Cancer			Cancer adjacent		
	Positive	Negative	Total	Positive	Negative	Total
SC	17	19	36	20	17	37
GC	13	10	23	14	8	22
G-SC	4	1	5	4	1	5
NDC	4	2	6	4	2	6
Total	38	32	70	42	28	70

Table 5. The distribution of HLA- A24 in different pathological types of lung cancer (n)

Pathologic type	Cancer			Cancer adjacent		
	Positive	Negative	Total	Positive	Negative	Total
SC	17	19	36	19	18	37
GC	13	10	23	12	10	22
G-SC	1	4	5	2	3	5
NDC	4	2	6	4	2	6
Total	35	35	70	37	33	70

4.2 The distribution and absence of HLA-A1, A2 and A24

The antigen that HLA class I gene codes is distributed in the human body extensively. During tumor immune process HLA class I antigen plays an important role in processing and presenting MAGE-A3 antigen. That is the basic point of tumor cell immune response inducement and adjustment. Many studies reported that HLA class I antigen was absent in many types of tumor[9]. Presently it has been reported that HLA-A1, A2, A3, A24, A29, B18, B35, B40 and B44 can be particularly combined with different MAGE-A3 antigenic determinant (epitope) respectively[10-15]. Among them, the HLA-A2 and HLA-A24 have very high distribution level in China[16]. With DNA typing method, our experiment results showed that for the distribution of HLA-class I molecule in tumor tissues the positive rates of HLA-A1, HLA-A2 and HLA-A24 were 4.28% (3/70), 54.28% (38/70)

and 50.0% (35/70), respectively. However, in tumor adjacent tissues the positive rates of HLA-A1, HLA-A2 and HLA-A24 were 4.28% (3/70), 60.0% (42/70) and 52.86% (37/70), respectively. The total positive rate of HLA-A1, A2 and A24 was 80.0% (56/70) in lung cancer patients. The result was in accordance with previous reports. This indicated that the antigenic determinant (epitope) (that can combine specifically with HLA-A1, A2 and HLA-A24 antigen) should be an ideal candidate for tumor specific vaccine.

In addition, our statistical results showed that there was no significant difference between lung cancer and cancer adjacent of these three alleles (P = ns). No significant differences were in different pathological types and different pathological stages of lung cancer (P = ns). This reminded that the lung cancer had all kinds of absence of HLA-A antigens but its gene structure was stable. Methods should be taken to induce these alleles' re-expression: such as interferon-γ[17], 5-aza-2′-deoxycytidine[18], even virus like newcastle disease virus[19].

5 Conclusion

Our research showed that the positive rate of MAGE-A3 antigen protein expression was 49.21%; HLA-A1, A2 and A24 total distribution was 80%. About 39% patients had HLA-A1 or A2 or A24 distribution and MAGE-A3 antigen expression. That means about 39% lung cancer patients may be treated with tumor specific vaccine based on MAGE-A3 antigen.

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Antagonistic Mechanisms of Zinc on Male Reproductive Toxicity Induced by Excessive Fluorine in Rats

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Abstract: Objective. To explore the antagonistic mechanism of zinc on male reproductive toxicity induced by excessive fluorine. **Methods.** A total of 30 male Wistar rats were randomly allocated into three groups: control group, fluorine group, and fluorine plus zinc group. Then the animals of three groups were fed deionized water supplemented with NaF or/and $ZnSO_4$ for 6 weeks. The levels of serum testosterone (T), superoxide dismutase (CuZn-SOD), lactate dehydrogenase (LDH), and Fas expression in spermatogenic cells were detected. **Results.** The levels of T in fluorine plus zinc group significantly increased compared to control group, and fluorine group, respectively ($P<0.05$). The activity of CuZn-SOD in control group and fluorine plus zinc group significantly increased compared to fluorine group ($P<0.005$). Compared to control group, the activity of LDH in testis in fluorine group decreased ($P<0.05$). Fas expression in fluorine group was significantly increased compared to control group and fluorine plus group ($P<0.001$). Fas expression in fluorine plus zinc group was increased ($P<0.05$) compared to control group. **Conclusion.** Appropriate zinc can antagonize male reproductive toxicity of fluorine on molecular level by antagonizing lipid peroxidation, influencing reproduction endocrine, activity of enzyme, and Fas expression. [Life Science Journal. 2006;3(2):32-34] (ISSN: 1097-8135).

Keywords: fluorine; zinc; male reproductive toxicity; antagonistic mechanism; rat

Abbreviations: CuZn-SOD: superoxide dismutase; T: testosterone; LDH: lactate dehydrogenase

1 Introduction

It was indicated that zinc could antagonize male reproductive toxicity of excessive fluorine effectively[1-3]. Zinc could reduce absorption of fluorine, with the result of decreased fluorine in the body. Thus it antagonizes fluorotic toxity by improving the activity of CuZn-SOD and antagonizing lipid peroxidation[4]. There was few report about the antagonistic mechanism of zinc on male reproductive toxicity of excessive fluorine. The purpose of this study is to explore above question on molecular level and to provide reference data and scientific basis accordingly for prevention and treatment of fluorosis.

2 Materials and Methods

2.1 Animals

After one-week quarantine, thirty Wistar male rats weighted 55 to 65 grams were randomly allocated into three groups with ten rats each group: control group, fluorine group, and fluorine plus zinc group. All rats were provided by Henan Experimental Animals Center (Zhengzhou, Henan, China). The animals in control group, fluorine group, and fluorine plus zinc group were fed deionized water, solution of NaF (25 mg/kg), and solution of NaF (25 mg/kg) supplemented with $ZnSO_4$ (20 mg/kg), respectively. Each group was treated with solution of NaF or/and $ZnSO_4$ in the way of intragastric administration for six weeks.

2.2 Indexes of test

2.2.1 The level of testosterone in serum: Rats of the three groups were killed and blood serum was separated. The levels of serum testosterone were detected radioimmunochemically with ^{125}I-T kit. And this kit was provided by Beijing Beimiandongya Biological Technology Ltd.

2.2.2 Activity of CuZn-SOD in serum: Rats of the three groups were killed and blood serum was separated. The activity of CuZn-SOD was detected with SOD kit and colorimetric analysis method, and the kit was provided by Nanjing Jiancheng Biological Technology Ltd.

2.2.3 Activities of LDH in testis: Plasm of the testicle tissue was prepared, and the activities of LDH were examined by colorimetric analysis. The kit was provided by Nanjing Jiancheng Biological Technology Ltd.

2.2.4 Expression of Fas protein in testis: The levels of Fas protein expression in testis were detected immunohistochemically. Fas immunohistochemical kit was provided by Wuhan Boster Biologi-cal Technology Ltd, and the experiment was manipulated according to the direction. The process was operated as follows: One side of testis of rats was put into formaldehyde liquor and fixed for 48 h in normal temperature. Volume concentration of formaldehyde is ten percent. After deparaffinization, tissue sections were treated with 3% H_2O_2 for 10 min to eliminate endogenous peroxidase. These sections were then heated in 10 mM citrate buter (pH 6.0) for 5 min in a microwave oven three times to facilitate antigen retrieval and were incubated with BSA for 20 min at room temperature. Subsequently, the sections were incubated with rabbit anti-mouse Fas monoclonal antibody at 4°C overnight. Thereafter, the sections were incubated with biotinylated goat anti-rabbit IgG for 20 min and were incubated with SABC for 20 min at room temperature. The immunoreaction was visualized with di-aminobenzidine tetrahydrochloride (DAB). The sections were lightly counterstained with hematoxylin.

2.3 Statistical analysis

One-way analysis of variance (ANOVA) and non-parametric tests were used in the statistical test (SPSS13.0). There was significant difference when α was 0.05. Differences were considered significant when $P<0.05$.

Table 1. T level and CuZn-SOD activity of each group

Types of group	*n*	T (ng/dl)	CuZn-SOD (U/ml)
Control	10	22.40	37.50
Fluorine	10	16.00$^{\triangle}$	20.10$^{*\triangle}$
Fluorine plus zinc	10	38.20*	36.30
H		13.05	21.30
P		0.011	0.000

Note: * Compared with control group, $P<0.05$; $^{\triangle}$Compared with fluorine plus zinc group, $P<0.05$

3 Results

Table 1 showed the levels of T and activity of CuZn-SOD in each group. The levels of T in fluorine plus zinc group significantly increased compared to control group, and fluorine group, respectively ($P<0.05$). Activity of CuZn-SOD in control group and fluorine plus zinc group significantly increased compared to fluorine group ($P<0.005$). Table 2 showed the activity of LDH and the level of Fas expression in every group. Compared to control group, the activity of LDH in testis in fluorine group decreased ($P<0.05$). Fas expression in fluorine group significantly increased compared to control group and fluorine plus zinc group ($P<0.001$). Meanwhile, Fas expression in fluorine plus zinc group increased ($P<0.05$) compared to control group.

Table 2. LDH activity and Fas expression of each group

Types of group	*n*	LDH (U/g Prot)	Fas(%)
Control	10	1329.75±814.97	7.65
Fluorine	10	07.34±885.66*	36.65$^{*\triangle}$
Fluorine plus zinc	10	1220.46±812.24	15.35*
H		2.841	42.41
P		0.035	0.000

Note: * Compared with control group, $P<0.05$; $^{\triangle}$Compared with fluorine plus zinc group, $P<0.05$

4 Discussion

Six weeks later, experiment, the level of T, the activity of CuZn-SOD, and LDH decreased. Fas protein expression in germ cells increased. Thus all above indicated that model of fluorosis rats was successfully established.

Zinc plays an important role in synthesis, secretion, and metabolism of hormone. Yu[5] reported that the serum T in zinc deficiency group significantly decreased compared to that in control group. But it recovered to normal after supplementing zinc. Takihara[6] reported that it was better to cure male sterility and improve activity of sperm mobility using zinc and male hormone together. These results indicated that the level of testosterone in fluorine plus zinc group was higher than those of control group and fluorine group, and it was lower in fluorine group than that in control group. This explained that appropriate zinc make T increase by antagonizing reproductive endocrine disruptance of fluorine. Zinc is the critical component of CuZn-SOD. It could antagonize fluorosis by improving activity of CuZn-SOD and antagonizing lipid peroxidation, lessening damage induced by free radicals. This results showed that the activity of CuZn-SOD in fluorine group was lower than control group and fluorine plus zinc group. There was no significant difference of the activity between control group and fluorine plus zinc group. This indicated that zinc could antagonize fluorosis in the way of improving activity of CuZn-SOD and antagonizing lipid peroxidation. Zinc is important in maintaining functions of many enzymes because it is the component of them in the body and it has something with structure of enzymes. It was reported that appropriate zinc could improve the activity of LDH[7], which

was proved in this experiment. This experimental results showed that the activity of LDH in fluorine group significantly decreased compared to control group and fluorine plus zinc group. That's why the excessive fluorine could decrease the activity of LDH, while increased it after supplementing zinc. Therefore, zinc could antagonize reproductive toxicity of fluorine by influencing the activity of LDH.

Fas is a type I membrane protein that belongs to the tumor necrosis factor (TNF)/ nerve growth factor receptor family and mediates apoptosis upon binding to Fas ligand, a type II membrane protein which is a member of the TNF family[8-10]. Fas is the apoptosis-inducing gene[11]. When cell apoptosis improves, fas expression improves too. Zinc is apoptosis-restraining gene which can prevent many factors from apoptosis. Nodera M[12] reported that zinc deficiency induced apoptosis in germ cells. Li[13] reported that appropriate zinc made apoptosis in germ cells siganificantly. In this experiment, Fas protein expression in fluorine group was significantly higher than those in control group and fluorine plus zinc group, and it was significantly higher than that in control group. It was obvious that fluorine could improve Fas protein expression in germ cells, which indicated that fluorine could induce apoptosis in germ cells. Zinc could reduce Fas protein expression. It may antagonize reproductive damage of fluorine probably by restraining apoptosis which induced by Fas gene in germ cells. Yet, after supplementing zinc, Fas protein expression was still higher than that of control group. The reasons were probably that antagonism of zinc on Fas protein expression and apoptosis in germ cells was infinite, or the dosage of zinc was not enough which could not obviously antagonize apoptosis induced by Fas protein expression. It is necessary to make more research to find the mechanism.

In a word, appropriate zinc could antagonize the reproductive toxicity induced by fluorine in male rats on molecular level by antagonizing lipid peroxidation, influencing reproduction endocrine, activity of enzyme and Fas expression.

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Effects of Carnoy's Solution and TCA on the Pouch Mucosa of Hamster

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Abstract: Objective. To investigate the injury of Carnoy's solution and trichloracetic acid (TCA) on the pouch mucosa of hamster, and provide evidence for these agents to be used in keratocyst treatment. **Methods.** 20 hamsters were employed as research objects, and divided into 4 groups randomly: Carnoy's group, 20% TCA group, 35% TCA group, and control group. The cheek pouches of hamster were treated with freshly prepared Carnoy's solution, 20% TCA and 35% TCA separately for 3 minutes. The specimens were taken immediately, and at 1, 3, 6, 12 weeks after operation. HE staining method, Van Gieson staining method, enzymohistochemistry and Transmission electron microscopy were used to study the effects of these agents on the mucosa. **Results.** Both Carnoy's solution and 35% TCA caused formation of fistula on pouch at the first week. Microscopic observation showed the epithelia formed an unorganized necrosis zone, and cells lose their morphous in Carnoy's solution group. The penetration depth was about 0.3 mm. In both 20% and 35% TCA group, the epithelia cell showed nuclear fragmentation or deliquescence, and internal structure lost, leading to the cell a vacuolar shadow. Arrangement of collagen fibers under epithelia was disordered; and fibroblast was disappearing in all test groups in the first week. From the 3rd week to the 6th week, the collagen fibers and fibroblast in all test groups were hyperplasia. **Conclusion.** Carnoy's solution and TCA have great penetrating power on the mucosa of cheek pouch, and they can destroy the epithelia and subcutaneous tissues. If these solutions could be for keratocyst therapy, they might cure the keratocyst or decrease recurrence rate. [Life Science Journal. 2006;3(2):35-40] (ISSN: 1097-8135).

Keywords: Carnoy's solution; TCA; hamster; cheek pouch; keratocyst

Abbreviations: HE: hematoxylin and eosin; TCA: trichloracetic acid

1 Introduction

Keratocyst of jaws is a common disease in oral and maxilloficial surgery. One of the major problems is the recurrence following the surgical treatment. To reduce the recurrence rate, attempts have been made by improving surgical techniques, such as removal of super-adjacent mucosa, smoothing of the osseous wall of the cystic cavity, resection of neighboring parts of the mandible, tanning of the epithelial lining of the cyst with Carnoy's solution and marsupialisation[1]. The results showed resection was found to have the lowest recurrence rate (0%) but the highest morbidity rate[2]. Simple enucleation was reported to have a recurrence rate of 17% to 56%. Simple enucleation combined with adjunctive therapy, such as the application of Carnoy's solution was reported to have recurrence rates of 1% to 8.7%[2,3].

As fixative solution, Carnoy's solution and TCA have great penetrating power on tissue. As an adjunctive therapy in treatment cyst, they can reduce the recurrence rate. But we did not know clearly: what is the mechanism of Carnoy's solution or TCA on the epithelia of cyst? Could Carnoy's solution or TCA destroy the secondary cyst that resided in fibrous capsule wall? How is the response of epithelia of cyst to the Carnoy's solution or TCA? To answer these questions, we took cheek pouch of hamster as epithelial model of keratocyst, to explore the mechanism of Carnoy's solution and TCA on the epithelia of keratocyst.

2 Materials and Methods

2.1 Experimental animal

20 hamsters were employed as research objects (provided by Institute of Biological Product, Wuhan), and were divided into 4 groups randomly: Carnoy's group, 20% TCA group, 35% TCA group, and control group.

2.2 Management method

Hamsters were anesthetized with pentobarbital sodium 45mg/kg. Then the cheek pouches of hamsters were exposed and treated with fresh prepared Carnoy's solution, 20% TCA and 35% TCA sepa-

rately for 3 minutes. The cheek pouches of hamsters in control group was treated with physiological saline. The specimens were taken in 0, 1, 3, 6, 12 weeks after operation.

2.3 General observation

After management with Carnoy's solution and TCA, the colorations and texture of cheek pouch were observed.

2.4 Epithelial changes

The specimens of cheek pouch were fixed with 10% formalin solution for 24 hours, then flushed with lotic water, dehydrated with gradient alcohol, and immersed in wax and embedded. 5 μm thickness slices were stained with HE. Observation was carried out under light microscope. The penetration depth of Carnoy's solution was detected with micrometer in 10×10 fields of view.

2.5 Collagen fiber observation

In order to observe the changes of collagen fiber below epithelia, Van Gieson staining[4] method was used in present study.

2.6 Enzymohistochemistry

7 μm thickness cryostat sections were made by freezing microtome. Then put the sections into dye vat, and added effective solution 100 ml (0.05 M acetic acid buffer solution 95 ml, β-sodium glycerophosphate 0.5 g, lead acetate 0.5 g, 5% magnesium chloride 5 ml, pH 5.0 – 5.2), 37 ℃, incubation for 80 min. In negative control group, β-sodium glycerophosphate was substituted by distilled water. After that the sections were put into 2% acetic acid solution for 1 min, and put them into fresh prepared 1% ammonium sulfide solution for 1 min in turn and flushed with distilled water behind every step. Then dehydrated with gradient alcohol, cleared with xylene, and mounted with optical gum, the sections were observed under light microscope.

2.7 Transmission electron microscopy

Specimens of cheek pouch taken in the 1st week and the 3rd week after operation were examined by transmission electron microscope. The specimens were fixed with 2.5% glutaraldehyde solution, dehydrated with gradient alcohol, and embedded with ethoxyline resin. Ultrathin sections were made, stained with uranyl acetate, and observed by transmission electron microscope.

3 Results

3.1 General observation

The mucosa of cheek pouch had obvious changes in appearance and texture after treatment with Carnoy's solution and TCA solution. In Carnoy's solution group, the color of mucosa of cheek pouch became black, the texture and elasticity lost in earlier stage. In TCA group, the color became white, especially the mucosa treated with 35% TCA. A week later, the mucosa became necrotic and ulcerative. There were 4 hamsters' cheek pouches formed fistulation because of exfoliation of ulcerative tissue (2 cheek pouch 2 hamsters for Carnoy's solution group and 35% TCA group respectively). 3 weeks after operation, the ulceration of mucosa had been recovered. From the 6th week the mucosa of cheek pouch had returned to their normal thickness and elasticity.

3.2 Epithelial changes (Figrues 1,2)

The mucosa of cheek pouch was keratosic stratified squamous epithelia. The thickness was about 3 – 5 layers cell with tenuis stratum corneum. In Carnoy's solution group, the epithelia turned black and the architecture could not be identified. According to the color change, the penetration depth was detected about 0.25 mm. In TCA group, the architecture was clear, and no obvious change was found. One week later, the epithelia formed an unorganized zone of necrosis, and cells lost their shape in Carnoy's solution group. The black change reached to the muscular layer with its depth about 0.3 mm and its circumscription was clear. Under the zone of necrosis, the evident inflammatory cell infiltration was found, even the inflammatory granulation tissues were showed. In TCA group, the epithelia cell showed nuclear fragmentation or deliquescence, and internal structure lost, made the cell to be a vacuolar shadow. There were no significant differences in epithelial between 20% TCA and 35% solution.

3 weeks after operation, the epithelia of cheek pouch recovered and architecture was clear in all test groups. The thickness of epithelia was 3 – 5 layers of cell. But in some areas, the thickness reached to 10 layers of cell. 6 weeks later, the epithelia had returned to normal, and there was no significant difference compared with normal epithelia of cheek pouch.

3.3 Changes of collagen fibers (Figures 3,4)

The fresh collagen fibers under epithelia showed normal architecture in all test groups, and showed red with Van Gieson stain. One week later, collagen fibers lost their normal architectures, such as disordered arrangement, and broken or even disappeared fibers in Carnoy's solution group. In TCA group, the arrangement of collagen fibers was irregular, and fibroblast was disappeared. It showed salmon pink in Van Gieson stain. 3 weeks later the collagen fibers and fibroblast in all test groups became hyperplasic. The arrangement of collagen fibers was compact. These changes made

the proper lamina to be augmented. From the 12th week, collagen fibers and fibroblast gradually tended to become normal.

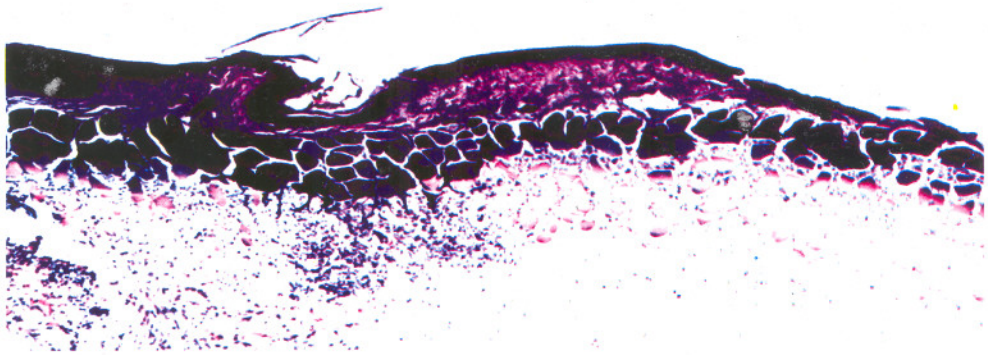

Figure 1. Carnoy's solution group at first week after operation. The figure showed necrosis of epithelia and penetration into muscular layer. (HE×100)

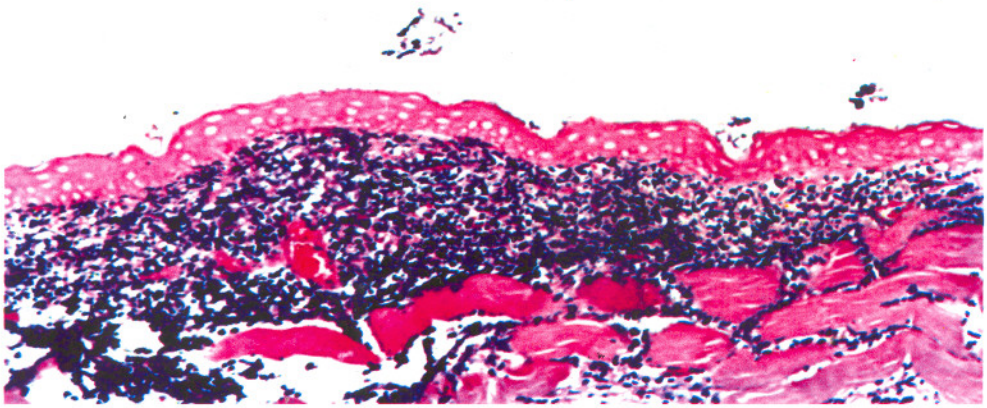

Figure 2. 35% TCA group at first week after operation. The figure showed disappearance of cellular structure, and the vacuolar shadow of the cells. (HE × 100)

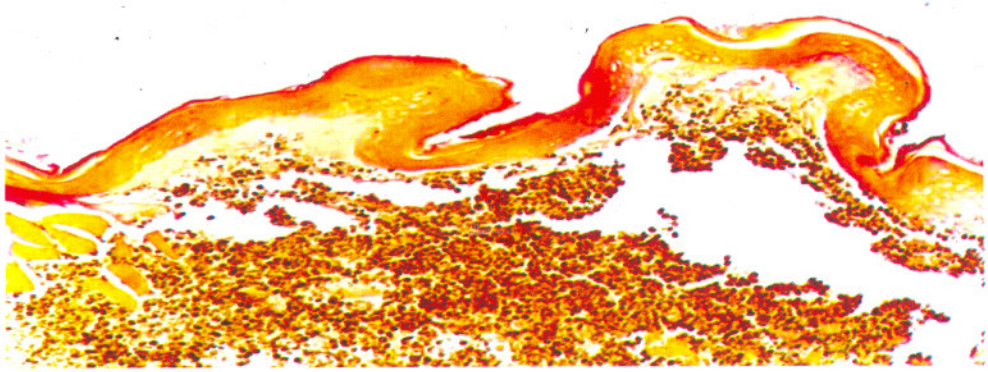

Figure 3. Carnoy's solution group at first week after operation. The figure showed disordered arrangement of collagen fibers, the broken or even disappeared collagen fibers. (VG × 200)

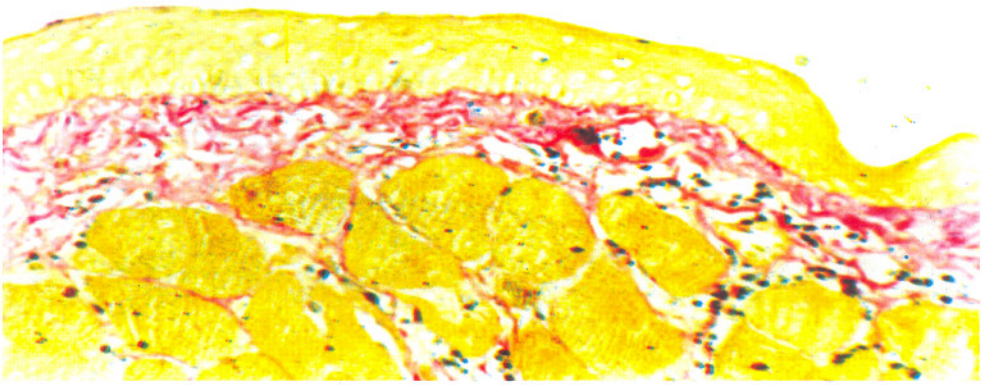

Figure 4. 20% TCA group at first week after operation. The figure showed disappearance of fibroblast. (VG × 400)

3.4 Enzymohistochemistry (Figures 5,6)

No enzyme activity in epithelial cells was found in all test groups until 3 weeks after operation; the positive was showed as yellow stain in epithelia of cheek pouch.

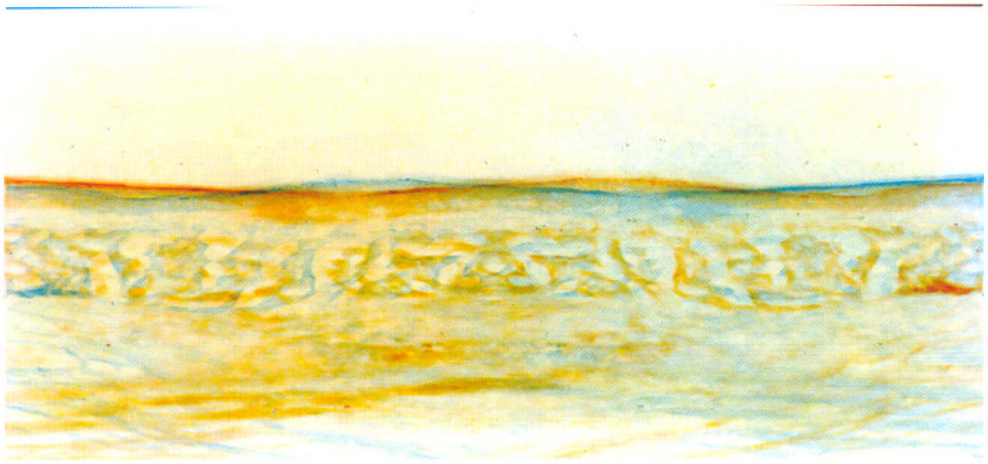

Figure 5. The specimen of Carnoy's solution at immediately time after operation. It showed disappearance of enzymatic activity at epithelial cell. (×200)

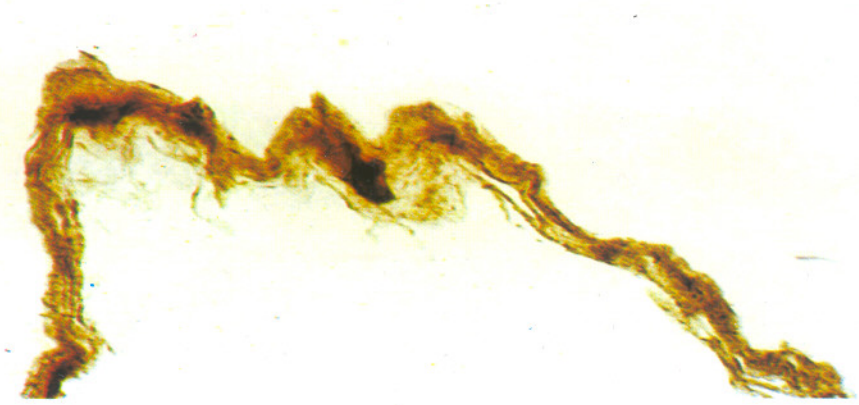

Figure 6. The specimen of 35% trichloracetc acid group 3 weeks after operation. It showed enzymatic activity at epithelial cell. (×100)

3.5 Transmission electron microscopy (Figures 7,8)

One week after operation, the epithelia of cheek pouch in Carnoy's solution group showed the increased electron density area was unorganized; cellular organelle could not be identified; basement membrane was discontinuous; desmosome and hemidesmosome disappeared. Leakage or vacuole was found in proper lamina; and fibrous structure could not be distinguished. The epithelial changes in TCA group were similar to that of epithelia in Carnoy's group. But collagen fibers could be recognized. There were no differences between 20% TCA group and 35% TCA group. After 3 weeks, the ultrastructure of epithelial cells could be recognized. In all test groups, the chondrosome in epithelial cell was swelling, the crista was short or disappeared, the heterochromatin was visible in nucleus; desmosome and hemidesmosome were recovered, and basement membrane was integrated. The difference between 20% TCA group and 35% was not significant.

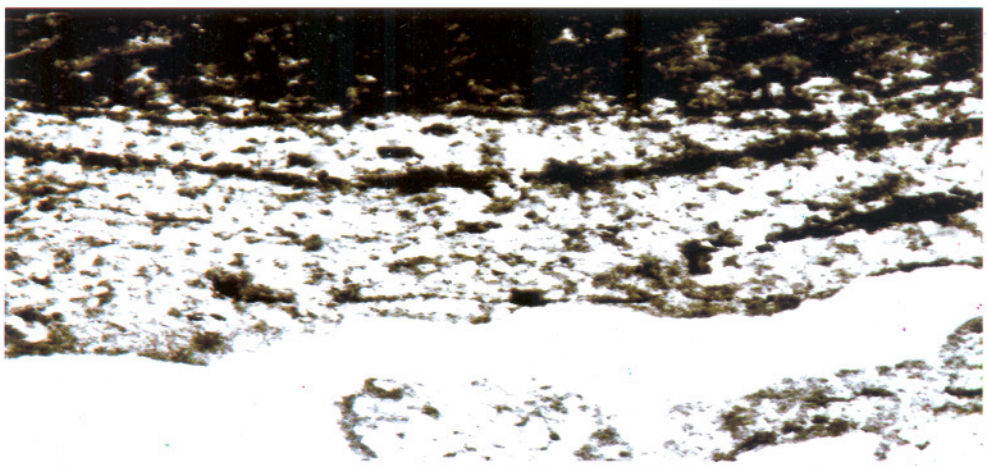

Figure 7. Carnoy's solution group at first week after operation. The figure showed ultrastructure could not be identified. (TEM × 10,000)

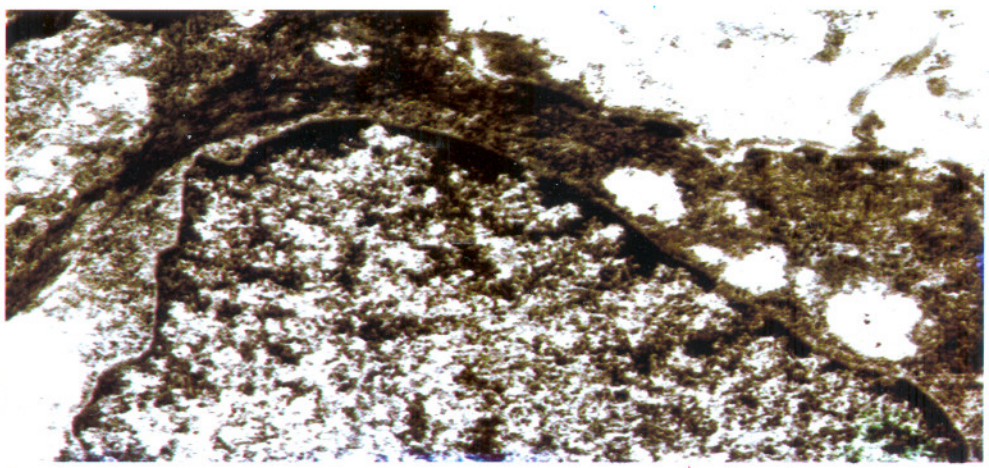

Figure 8. 20% TCA group 3 weeks after operation. The chondrosome of epithelial cell was swelling, and crista was short or disappears. (TEM ×15,000)

4 Discussion

Both Carnoy's solution and TCA are chemical materials with causticity. And Carnoy's solution was usually used as fixative solution, and TCA was used as peeling solution[5]. Living tissue would be damaged with these chemical solutions. The degree of injury was relevant to: ① chemical strength of reagent; ② quantity of reagent; ③ exposure chamber and time; ④ strength of penetration; ⑤

mechanism of action[6].

The mucosa of cheek pouch is cuticular stratified squamous epithelia, whose thickness is 3 – 5 layers of cells. And it is similar to epithelia of keratocyst in origin. So we took cheek pouch as model of keratocyst to investigate the mechanism of Carnoy's solution and TCA in treatment of cyst.

Penetration is one of important factors of Carnoy's solution leading to tissue damage. Some studies showed depth of penetration of Carnoy's solution on nerve was releated to time. But the penetrating process was not continuous because of barrier function of perineural epithelia[7]. In this study, we observed Carnoy's solution's action on mucosa, which resulted in the formation of fistula in cheek pouch and for 3 min the depth of penetration was about 0.3 mm. The difference in depth of penetration on different tissue may be related to their structure.

Some studies showed degree of skin destruction made by TCA was not linear with TCA concentration. However, high concentration of TCA might cause strong reaction and severe clinical pathological changes. The higher concentration is, the deeper the penetration[7,8]. In this study, the results of 20% TCA and 35% were different. In 35% TCA group, the color change of cheek pouch was greater than that of 20% TCA, and fistula was formed in cheek pouch. These results further demonstrated that effects of 35% TCA on mucosa were greater than 20% TCA.

Proper lamina of mucosa is connective tissue that was mainly constituted by collagen fibers. It has important influence on the epithelial cells. The mucosa as well as its collagen fibers in proper lamina would be damaged when treated with those solutions with penetrating power, such as Carnoy's solution and TCA. The results were supportive for Carnoy's solution and TCA to treat keratocyst, for secondary cyst that resided in fibrous capsule wall would be eliminated by their penetrating power.

Reepithelialization of treatment areas is another question we should pay attention to. The regenerative cells of treatment areas came from the adjacent tissues not the injured zone. It reminded that we should treat lining epithelia of keratocyst thoroughly when we use these solutions to treat keratocyst. Otherwise, any residual epithelia may lead to recurrence of keratocyst.

The present study demonstrated that Carnoy's solution and TCA had great penetrating power on the mucosa of cheek pouch, and be destroyed the epithelia and subcutaneous tissue. If we use these solutions to treat keratocyst, they maybe cure the keratocyst or decrease recurrence rate after operation.

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Antiangiogenic Effect of Cyclophosphamide by Metronomic Chemotherapy

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Abstract: Objective. To observe the effect of metronomic chemotherapy with cyclophosphamide (CTX) on tumor growth and angiogenesis on mouse Lewis lung carcinoma (LLC) model. **Methods.** LLC cells were injected subcutaneously into C57BL/6 mice on day 0. Mice were randomly divided into metronomic group, MTD group and control group. Therapies were given to the groups from the 6th day. Mice weight and tumor diameter were measured every other day. Then tumors were weighed. Microvessel count and proliferating index of tumor cell were performed by immunohistochemical staining with anti-CD_{31} antibody and anti-PCNA antibody. The histological characteristics of the tumor were detected by microscopy. **Results.** Tumor growth and angiogenesis were inhibited by CTX significantly in the metronomic group, which has better efficacy and lower toxicity than maximum tolerated dose (MTD) group. The average weight of the tumors in control group, metronomic group and MTD group were (3.72 ± 0.60) g, (1.85 ± 0.38) g and (1.73 ± 0.41) g, the average microvessel count were 26.38 ± 2.56, 15.09 ± 3.03 and 23.51 ± 2.78, PCNA index were 83.88 ± 3.72, 81.60 ± 4.21 and 71.80 ± 3.86, respectively. Necrosis was increased significantly in the metronomic group. **Conclusion.** CTX inhibits tumor growth and angiogenesis in mouse LLC model significantly. The inhibition may be correlated with the anti-angiogenic effect of CTX. [Life Science Journal. 2006;3(2):41-44] (ISSN: 1097-8135).

Keywords: metronomic chemotherapy; Lewis lung carcinoma; anti-angiogenesis

Abbreviations: CTX: cyclophosphamide; LLC: Lewis lung carcinoma; MTD: maximum tolerated dose

1 Introduction

One of the great advances in cancer treatment during last half of the 20th century was the development and utilization of chemotherapeutic drugs. These compounds have demonstrated anti-tumor efficacy through their damaging action against cellular DNA, which prevents proliferation and, frequently, drives the cell to its death. Since such effect is only exerted on a fraction of tumor cells, the administration of higher drug doses are required to achieve better clinical results. However, the application of the highest drug doses tolerated by the patient brings about the problem of drug toxicity. Thus, it is mandatory to establish rest periods, which not only allow regrowth of tumor cells but also growth of selected therapy-resistant clones. After the successful first cycles of treatment, tumors acquire resistance to the chemotherapeutic drugs. Thus, the growth of those resistant cells involve the development of more aggressive and malignant tumors.

In order to avoid the problems caused by traditional chemotherapeutic treatments, several researchers, recently began to search for new modalities of drug administration oriented towards a more efficient and non-toxic antitumoral and/or antimetastatic therapy. They found that some cytotoxic drugs, when given in low dose, high frequent mode, they could significantly inhibit angiogenesis in the tumor bed, resulting in preventing tumor growth because of ischemia. To be distinguished from traditional MTD, they called it metronomic chemotherapy[1], and its therapy target is endothelial cells.

The objective of this study was to investigate the antiangiogenic effect of cyclophosphamide (CTX) with metronomic chemotherapy on LLC mouse models, and to determine the toxicity of the treatment.

2 Materials and Methods

2.1 Animals, reagents and tumor specimens

30 male C57BL/6 mice (provided by Henan Experimental Animal Center) were used, weighing from 18 g to 22 g. CTX, in the injection power form, was the production of Jiangsu Hengrui Medical Corporation. LLC cells were preserved in C57BL/6 mouse and provided by Henan Medical

Science Institute.

2.2 Experimental models and therapeutic schedules

LLC cells were injected subcutaneously into the right oxter of C57BL/6 at 1×10^6/0.2 mL on day 0, and distributed randomly as follows: group Ⅰ control mice ($n=10$) were injected i.p. with saline once per day from day 6 until animals were killed; group Ⅱ metro group mice ($n=10$) were injected i.p. with CTX (20 mg/kg body weight) once per day, from day 6 until animals were killed; and group Ⅲ MTD group mice were injected i.p. with CTX (150 mg/kg body weight) once every other day over 6 days from day 6, followed by 2 weeks interval (21-day was a cycle).

Tumors were measured every other day from day 6 by a caliper. Tumor volumes were calculated as follows: $V=0.52\ (ab^2)$, where V = volume (mm^3), a = largest diameter (mm) and b = small diameter (mm) perpendicular with a. Animals were weighed every other day from day 6. On day 20, all animals were killed and tumors were excised. Tumors were weighed, fixed in 10% formalin and processed for immunohistochemical analysis. Paraffin blocks were cut to 4 μm sections and stained with hematoxylin and eosin (HE) for histological examination and with anti-CD_{31} and anti-PCNA antibody for assessment of microvessel density and tumor cell proliferation index, respectively.

2.3 Statistical analysis

We used statistical software package SPSS 12.0 to analyze the experiment results. The level of significance was set at $P<0.05$.

3 Results

3.1 Tumor growth assessment

Compared with control group, significant growth delays of tumor were observed in metro group, while in MTD group, the tumor volume decreased immediately after therapy, but increased during the rest periods. The growth curve was shown in Figure 1.

3.2 Body weight assessment

As for the body weight, steady increases were showed in control group and metro group, on the contrary, there were weight loss in the MTD group after therapy and weight increase during the rest periods. The results were showed in Figure 2. After mice were killed and tumors were excised, tumor weight in control group, metro group and MTD group were (3.72±0.60) g, (1.85±0.38) g and (1.73±0.41) g, respectively. Statistical differences were shown between control group and the other two groups.

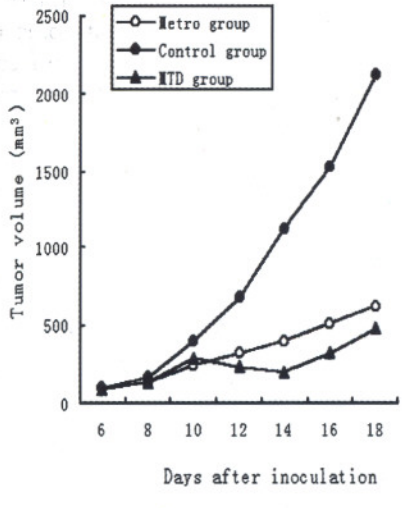

Figure 1. Curves of tumor volume

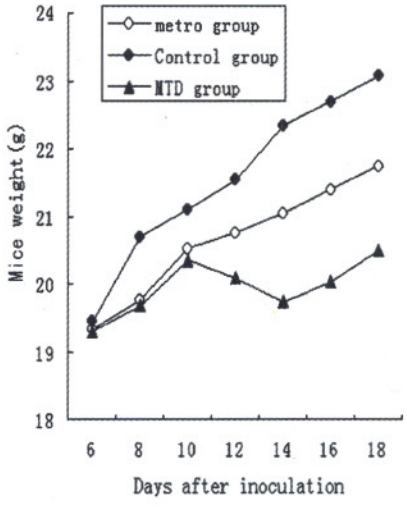

Figure 2. Curves of mice weight

3.3 Immunohistochemical results (Table 1)

On histological examination, no significant difference in morphology was found between tumors of the control group and therapy groups, but there were many necrosis areas in the metro group. Measurement of microvessel count and PCNA index showed differences among the groups of treatment. Tumors belonging to animals of the control group

showed the highest microvessel count and PCNA index. Those from animals submitted to MTD chemotherapy had intermediate microvessel count and the lowest PCNA index, whereas the lowest microvessel counts and intermediate PCNA index were measured in tumors from animals received metronomic schedule.

Table 1. Results of immuneohistochemical($\bar{\chi} \pm S$)

Group	Cases	Microvessel count	PCNA index
Control group	10	26.38 ± 2.56	83.88 ± 3.72
Metro group	10	$15.09 \pm 3.03^*$	81.60 ± 4.21
MTD group	10	23.51 ± 2.78	$71.80 \pm 3.86^*$

Note: $^*P<0.01$ compared to control group

4 Discussion

Historically, chemotherapy regimens have been controversial: which way should the scale be tipped between efficacy in tumor killing and lack of toxicity? On one hand, chemotherapeutic drugs could disrupt the DNA of tumor cells, breaking their replication and finally killing them, the befitting corollary being "the higher the dose the better"[2]. On the other hand, toxicity is found at several organ sites, which not only diminishes quality of patient life but also conspires against a good resolution of the cancer treatment, adding illness to that which already exists[3]. The introduction of maximum tolerated doses in usual treatment protocols made it necessary to impose intervals between cycles of therapy. During such intervals, recovery of "good" cells is frequently accompanied by recovery of "bad" cells, i.e. tumour cells resistant to the drug-resistant tumor cells. The possibility of finding a treatment modality that avoids toxicity without diminishing effectiveness is still a matter for study and discussion. The experimental findings of Browder[4], Klement[5,6] and Man[7] and colleagues successfully introduced a novel strategy for cancer treatment. The novel component comprised a cell target switch, which now aims towards the genetically stable tumor endothelial cells, along with a change in the schedule and dose of drug administration, thereby introducing metronomic chemotherapy[1].

The experimental models were designed to resemble the clinical situation of a patient with a recently detected tumor (not too large, but large enough to be easily detected) who begins therapy immediately after being diagnosed. Metronomic therapy began after 6 days of the tumor challenge. Our results showed that tumor growth and angiogenesis were significantly inhibited by low dose CTX. Tumor weight in metronomic group was statistically lower than that in control group. Body weight in metronomic and control groups increased steadily, which suggested that the toxicity of metronomic schedule was minimal. Though the short-term antitumor of MTD chemotherapy was effective, it accompanied with significant weight losses and tumor regrowth during the intervals. The lowest microvessel count and the lowest PCNA index were in metro group and MTD group, respectively. So we supposed that MTD schedule produced the effect mainly through inhibiting tumor cells growth or inducing cells death; while metronomic therapy induced tumor cells ischemia and necrosis mainly through damaging vessels in the tumor bed and its antitumor effect was related with its ability of decreasing microvessel count.

Other authors have demonstrated clearly the antiangiogenic nature of several drugs administered by metronomic dosing. Moreover, the combination of such treatment with specific antiangiogenic reagents increased significantly the observed antitumour effect of metronomic dosing[4-6,8]. In fact, literature dating back to the mid-1980s showed that virtually almost all the traditional chemotherapies had antiangiogenic effects or antivascular effects in various *in vitro* and *in vivo* assays[9]. But the antiangiogenic effects of chemotherapy were masked and marginalized by the general chemotherapy. In this case, the long intervals between drug administrations were necessary for the patient to recover from the harmful side effects of both the MTD and the anti-angiogenic chemotherapy drugs. So, if we give drugs more frequently, such as once or more per week without extended breaks, there would be significantly less opportunity for repairing the damaged endothelium and the anti-angiogenic effects of the chemotherapy. This, of course, necessitates reducing the dosage of the drug administered with each injection.

In short, metronomic chemotherapy has many advantages, but its molecular mechanism is unclear and there are several significant challenges in clinical applications. Foremost among these is the current empiricism associated with determining the optimal dose and schedule for administration of chemotherapeutics. A second challenge is the prospect of delayed side effects, including secondary neoplasms. In particular, it should be clear about which chemotherapeutics are the most effective for metronomic regimens, what kind of combinations and sequences might be the best, and what mechanisms of resistance might develop for a long-term therapy.

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Microarray Analysis: Single Cell Gene Expression by GeneChip Protocol

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Abstract: The microarray technology is a new, powerful and useful tool for gene expression, clinical diagnosis, food safety control and other the biochemical researches. Using the microarray technology, more than 10,000 genes or proteins can be printed on one location. The supports can be silicon chips, nylon membranes or glass slides, etc. This article is giving a brief description of microarray protocol of GeneChip in the single cell gene expression as an example. [Life Science Journal. 2006;3(2):45-49] (ISSN: 1097-8135).

Keywords: DNA; GeneChip; microarrays; protein

1 Introduction

Microarrays are new methods. Using microarray technology more than 10,000 genes or proteins can be printed on a glass slide (MacBeath, 2000) and thousands of genes can be detected and analyzed in an array simultaneously by the microarray analysis. Microarray could be named as biochip, DNA chip, DNA microarray, gene array, gene chip, and protein array, etc. Microarrays have become a crucial component of gene expression and genotype research recently. Microarray technologies are powerful tools to measure the expression of many genes simultaneously.

The most important microarray is DNA microarray. For the DNA microarray, thousands of different DNA molecules (genes) are fixed on a support. The supports can be silicon chips, nylon membranes or glass slides. The DNA is printed, spotted, or actually synthesized directly onto the support. Each single-stranded DNA fragment is made up from four different nucleotides, adenine (A), thymine (T), guanine (G), and cytosine (C). During DNA molecule synthesis, A is the complement of T, and G is the complement of C. Therefore, the complementary sequence of A-C-G-T-T-G-C-A will be T-G-C-A-A-C-G-T. When two complementary sequences match to each other, such as the target DNA (immobile DNA) and the sample DNA (mobile DNA), cDNA, or mRNA, they will combine together (hybridize). The mobile DNA can be labeled with fluorescence as the mobile probe to detect gene expression level if there are complementary molecule sequences existing in the immobile slides.

Right ventricular hypertrophy and failure are prominent features in cyanotic congenital heart disease, tetralogy of Fallot. To detect the molecular mechanisms of right ventricular hypertrophy and to identify gene(s) involved in tetralogy of Fallot, Sharma and colleagues measured the differential gene expression using expression-based microarray technology on right ventricular biopsies from young tetralogy of Fallot patients who underwent primary correction. By using quantitative immuno histochemistry, expression of vascular endothelial growth factor, flk-1, and extracellular matrix proteins (collagens and fibronectin) as well as vessel counts and myocyte cell size was evaluated in TF patients in relation to age-matched controls. From these studies, they concluded that the upregulation of genes encoding vascular endothelial growth factor and extracellular matrix proteins were the key events contributing to right ventricular hypertrophy and stunted angiogenesis in patients with tetralogy of Fallot (Sharma, 2006).

Cross-validation represents a tool for reducing the set of initially selected genes to those with a sufficiently high selection frequency. Using cross-validation it is also possible to assess variability of different performance indicators (Qiu, 2006).

Cytoplasmic control of the adenylation state of mRNAs is a critical post-transcriptional process involved in the regulation of mRNAs stability and translational efficiency. The early development of Xenopus laevis is a major model to study this regulation. Graindorge et al used microarray method to identify mRNAs that were regulated by changes in their adenylation state during oogenesis and early development of the diploid frog Xenopus tropicalis.

The microarray data were validated using qRT-PCR and direct analysis of the adenylation state of endogenous maternal mRNAs during the period studied. They successfully identified more than 500 mRNAs regulated at the post-transcriptional level among the 3000 mRNAs potentially detected by the microarray (Graindorge, 2006).

Pterygium is an ocular-surface lesion that can decrease vision. In order to detect the genes that may play roles in pterygium pathogenesis, John-Aryankalayil et al analyzed the global gene expressions of pterygium. In John-Aryankalayil's studies, oligonucleotide microarray hybridization was used and the selected genes were further characterized by RT-PCR, Western blot, and immunohistochemistry, and comparisons were made with limbal and corneal tissues. Their results showed both novel and previously identified extracellular-matrix-related, proinflammatory, angiogenic, fibrogenic, and oncogenic genes expressed in human pterygium (John-Aryankalayil, 2006).

Using the tissue microarray technology with highly reliable method of fluorescent in situ hybridization, Dimova et al showed similar frequencies of epidermal growth factor receptor gains in different grade tumors, while EGFR amplification increased from grades 1 to 2 to 3 (Dimova, 2006).

Enzyme-linked immunosorbent assay (ELISA) microarray technology can simultaneously quantify levels of multiple proteins, which has the potential to accelerate validation of protein biomarkers for clinical use (Zangar, 2006).

As the microarray technology development, massive amounts of microarray images are produced. The storage and the transmission of the microarray images are significant important for the research and application of microarray technology. Lonardi and Luo proposed lossless and lossy compression algorithms for microarray images originally digitized at 16 bpp (bits per pixels) that achieve an average of 9.5 − 11.5 bpp (lossless) and 4.6 − 6.7 bpp (lossy, with a PSNR of 63 dB). The lossy compression was applied only on the background of the image, thereby preserving the regions of interest. The methods were based on a completely automatic gridding procedure of the image (Lonardi, 2006).

Diaz-Uriarte and Alvarez de Andres investigated the use of random forest for classification of microarray data using simulated and nine microarray data sets they showed that random forest has comparable performance to other classification methods, including DLDA, KNN, and SVM. Because of its performance and features, random forest and gene selection using random forest should probably become part of the standard method for class prediction and gene selection with microarray data (Diaz-Uriarte, 2006).

Theoretical considerations of protein microarrays were done in the 1980's by Roger Ekins and colleagues (Ekins, 1989;1991;1994;1999).

Oligo GEArray is a new oligonucleotide-based gene expression array from SuperArray Bioscience Corporation. It combines current oligo-based array design with SupperArray's proven nylon membrane based array technology.

Using microarray analysis, a typical experimental protocol could be:

↓

Isolating RNA

↓

Converting the RNA samples to labeled cDNA via reverse transcription

↓

Hybridizing the labeled cDNA to identical membrane or glass slide arrays

↓

Removing the unhybridized cDNA

↓

Detecting and quantitating the hybridized cDNA

↓

Data analysis with/without software

2 Gene Blots (96-well size) Making Protocol in the Single Cell Gene Expression

The following gives the brief steps for the gene plots making protocol in the single cell gene expression experiment, as the reference for the researchers.

I. Making DNA blots

1. Add 1 μl plasmid with gene into 1.5 ml eppendorf tube
2. Add 10 μl DEPC H_2O
3. On ice 5 min
4. Add 20 μl competent cells
5. On ice 30 min
6. 42 ℃ 50 sec
7. On ice 3 min
8. Add 1 ml LB medium
9. 37 ℃ 1 h
10. Add 100 μl of above cell suspension to agar plate with ampicillin
11. Spread
12. 37 ℃ over night
13. Pick one colony
14. Grow in 3 ml LB medium with ampicillin over night

15. Spin 10 min at 10,000 rpm
16. Remove supernatant away
17. Suspend in 250 μl P1 buffer (Invitrogen DNA mini-purification kit)
18. Vortex
19. Add 250 μl P2 buffer (Invitrogen DNA mini-purification kit)
20. Add 350 μl N3 buffer (Invitrogen DNA mini-purification kit)
21. Spin 1 min
22. Pour to column (Invitrogen DNA mini-purification kit)
23. Spin 1 min
24. Wash with 750 μl PE buffer (Invitrogen DNA mini-purification kit)
25. Spin 1 min
26. Through away pass through
27. Spin another 1 min and transfer column onto another new eppendorf tube
28. Add 50 μl DEPC H_2O
29. Spin 1 min
30. Take 1 μl, and add 100 μl DEPC H_2O
31. Read O D 260 nm
32. Calculate volume for digestion
33. Add purified P-DNA and endonuclease
34. 37℃ over night
    1) Prepare 0.8% agrose gel
    2) Run gel for 1.5 h 100 v
    3) Ethydine bromide stain
    4) Take picture to check the purity of the gene
35. Wet filter paper
36. Wet N-bond membrane
37. Lay the membrane on the blot machine
38. Add DNA clones (genes)
39. Suck 10 min
40. Cross linking the membrane

II. Making immuno-staining

41. Stepwise treat the tissue slides with xylene, ethanol, methanol, H_2O
42. Wash 10 min with running water
43. Dip in 0.1 M Tris-HCl for 5 min
44. Add 2% FBS on the slide and keep for 5 min
45. Add primary antibody and keep at 4 ℃ over night
46. Wash with 0.1 M Tris-HCl for 5 min
47. Keep in 2% FBS for 5 min
48. Add secondary antibody and keep for 1 h
49. Wash with 0.1 M Tris-HCl for 5 min
50. Keep in 2% FBS for 5 min
51. Keep in A/B reagent for 1 h
52. Add DAB reagent on the slide and keep for 10 min at room temperature
53. Wash with DEPC H_2O for 5 min
54. Soak in DEPC H_2O

III. Single cell gene expression

55. Take the slide out from DEPC H_2O
56. Add 100 μl proteinase K
57. Keep at 42℃ for 30 min
58. Rinse with DEPC H_2O
59. Make oligo-dT mix
60. Add 100 μl oligo-dT mix onto slide
61. Keep at room temperature over night
62. Wash off oligo-dT with 2 × SSC buffer
63. Soak in 2 × SSC buffer for 15 min
64. Dilute 10 × first buffer to 1 × first buffer
65. Add 100 μl of the 1 × first buffer onto the slide
66. Keep at room temperature for 30 min
67. Remove the 1 × first buffer
68. Add the 1 × first buffer reaction mixture 100 μl on slide
69. Keep at 37 ℃ for 90 min
70. Remove the 1 × first buffer reaction mixture
71. Soak in 2 × SSC buffer

IV. cDNA synthesis

72. Prepare electrode buffer
73. Add 20 μl electrode buffer into eppendorf tube
74. Pick positive standard cell
75. Add the positive standard cell into tube
76. Keep at 37 ℃ for 1 h
77. Add 50 μl phenol-chloform
78. Vortex
79. Spin at 14,000 rpm for 20 min
80. Transfer top layer to a new tube
81. Add 100 μl ethanol (100%) and 10 μl 3M NaAc and 0.5 μl tRNA
82. Keep at −80℃ over night

V. Loop expression

83. Spin at 14,000 rpm at 4 ℃ for 15 min
84. Remove ethanol and dry pellet for 20 min
85. Suspend pellet in 20 μl DEPC H_2O
86. Keep at 95℃ for 15 min
87. Add 22 μl 2nd strand DNA synthesizing mixture
88. Incubate at 14℃ for 4 h or over night
89. Make loop excision mixture
90. Add 350 μl for the above mixture into each 42 μl sample
91. Keep at 37℃ for 5 min
92. Extract with 400 μl phenol-chloroform
93. Spin at 14,000 rpm for 15 min

94. Remove top layer to a new tube
95. Add 1 ml ethanol (100%)
96. Keep at −80℃ over night
97. Spin at 14,000 rpm for 15 min

VI. Blunt ending

98. Remove ethanol and dry pellet
99. Suspend pellet in 17.5 μl TE buffer
100. Make blunt mixture
101. Add 7.5 μl of the above mixture into each tube
102. Keep at 37℃ for 15 min
103. Add 25 μl phenol-chloroform
104. Vortex
105. Spin at 14,000 rpm for 15 min
106. Remove top layer to a new tube
107. Add 55 μl ethanol and 7.7 μl 3 M NaAc
108. Keep at −80℃ over night

VII. RNA analysis and labeling

109. Spin at 14,000 rpm for 15 min
110. Remove ethanol and dry the pellet for 20 min
111. Suspend the pellet in 20 μl DEPC H_2O
112. Prepare RNA amplification buffer with 32P-UTP
113. Add 8.5 μl of the RNA amplification buffer into 2 μl cDNA
114. Keep at 37℃ for 4 h
115. Prepare denature gel
116. Run gel for 1.5 h at 100 v
117. Wash gel with cold 10% TCA for 4 times
118. Press dry gel by paper towel for 4 h or over night
119. Expose gel to X-ray film for 1-3 days
120. Develop X-ray film

VIII. Hybridization

121. Wet DNA blot with DEPC H_2O and 2 × SSC buffer
122. Prepare hybridization buffer
123. Prehybridize blots in 20 ml hybridization buffer at 43 ℃ for 2 h
124. Keep 32P-cRNA (from step 114) at 95 ℃ for 5 min to denature
125. Keep on ice quickly
126. Add the denatured 32P-cRNA into hybridigization tube
127. Hybridigize at 43℃ for 72 h
128. Wash blots with 2 × SSC buffer 4 times
129. Semi dry blots
130. Bleach phospho-image screen for 30 min
131. Expose the labeled DNA blot to phospho-image screen for 1 − 3 days
132. Run phospho-image in image machine
133. Down load to computer
134. Analyze image with image software

3 Discussion

The microarray technology is a useful tool for gene expression, clinical diagnosis, food safety control and other the biochemical researches. In a review article, Roy and Sen pointed that the cDNA microarray approach is an emergent technology in diagnostics and food safety test (Roy, 2006). Its values lie in being able to provide complimentary molecular insight when employed in addition to traditional tests for food safety, as part of a more comprehensive battery of tests. Gene-expression biomarkers measured by microarray method can be used to identify promising candidate caloric restriction mimetics that may be involved in determining human longevity (Spindler, 2006).

Gene expression is the essential characterization for organisms to adapt to changes in the external environment. The measurements of gene expression supply the information about the mechanism of organisms' living activities. The development of high-quality microarrays has allowed this technology to become a standard tool in molecular detection including cell toxicology. Several national and international initiatives have provided the proof-of-principle tests for the application of gene expression for the study of the toxicity of new and existing chemical compounds. In the last few years the field has progressed from evaluating the potential of the technology to illustrating the practical use of gene expression profiling in toxicology. The application of gene expression profiling to ecotoxicology is at an earlier stage, mainly because of the many variables involved in analyzing the status of natural populations. Nevertheless, significant studies have been carried out on the response to environmental stressors both in model and in non-model organisms. It can be easily predicted that the development of stressor-specific signatures in gene expression profiling in ecotoxicology will have a major impact on the ecotoxicology field in the near future. International collaborations could play an important role in accelerating the application of genomic approaches in ecotoxicology (Lettieri, 2006).

In the past decade, microarray technology has become a major tool for high-throughput comprehensive analysis of gene expression, genotyping and re-sequencing applications (Scaruffim, 2006). The industrial era of microarray will come soon. It will enhance the molecular biology development to a new level.

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Transfer and Expression of VEGF Gene in Neural Stem Cells

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Abstract: Objective. To construct retroviral vector containing vascular endothelial growth factor (VEGF) and observe its expression in rat neural stem cells. **Methods.** The recombinant retroviral vector pLXSN-VEGF containing VEGF gene was established and transferred to packaging cell line-pA317 to acquair virus. The titer was calculated by infection of NIH3T3 cells, then the recombinant virus was to infect neural stem cells (NSCs) isolated from the whole brain of embryonic day 14 (E14) SD rats. Transduced cells were cloned in G418 and expanded for analysis of VEGF transgene expression, VEGF mRNA, VEGF expression and its expression curve were detected and worked by using RT-PCR, immunocytochemistry and enzyme-linked immunosorbent assay (ELISA), respectively. **Results.** Retroviral vector pLXSN-VEGF was successfully established and it was proved by enzyme cutting and sequence analysis. The titer of retrovirus was determined to be 6.5×10^5 CFU/ml. RT-PCR and immunochemistry verified the expression of VEGF in neural stem cells. The peak of VEGF expression was showed 6-8 days after transfection by ELISA. **Conclusion.** Retroviral vector containing VEGF were successfully constructed. VEGF gene can be stably expressed in rat neural stem cells. [Life Science Journal. 2006; 3(2): 50 - 54] (ISSN: 1097 - 8135).

Keywords: NSCs; VEGF; retrovirus; transfection; transgene

Abbreviations: NSC: neural stem cell; VEGF: vascular endothelial growth factor

1 Introduction

Neural stem cells (NSCs) are the primary cell element for generation and maintenance of nervous system. Their essential biologic characteristics are: undifferentiation, deficiency of differentiation signal, self-renewal, potency to differentiate into neurons, oligodendrocyte and astrocyte[1]. NSCs may provide for cell replacement or gene delivery vehicles in neurodegenerative disease therapies[2], and target delivery and expression of growth factor transgenes such as vascular endothelial growth factor (VEGF) in NSCs to construct genetic engineering[3]. NSCs is an important direction in gene therapy of nervous system disease. The secreted glycoprotein VEGF is a potent and specific mitogen for vascular endothelial cells that is capable of stimulating angiogenesis during embryonic development and adult neurogenesis *in vivo*[4]. It has been reported that VEGF stimulated the expansion of neural stem cells. VEGF also acts as a trophic factor for neural stem cells *in vitro* and for sustained neurogenesis in the adult nervous system[5]. This experiment plans to construct a retroviral vector containing VEGF gene, transfer the gene into NSCs and detect the expression of it; and to provide experimental evidence for transgenic neural stem cell transplantation therapy of neurodegenerative diseases.

2 Materials and Methods

2.1 Materials

The plasmid pcDNA 3.1-VEGF contains a cDNA copy of the human VEGF gene, constructed in our laboratory using a pcDNA3.1 plasmid backbone (Invitrogen, Carlsbad, CA, USA). Pregnant SD rats were offered by Experimental Animal Center of Henan Province of China (Zhengzhou, China). Main reagents: restriction endonuclease *EcoR*I and *Xho*I, T4 DNA Ligase (TaKaRa); retroviral vector pLXSN (clontech); *E. coli* JM109 (TaKaRa); plasmid miniprep kit, Agarose Gel DNA fragment recovery kit, DNA extraction kit (Watson Biotechnologies, INC); Lipofectamine 2000 Liposome, B27, bFGF (Invitrogen); DMEM/F12, FBS (Hyclone); Nestin antibody, VEGF antibody (Boster).

2.2 Methods

2.2.1 Constuction of recombinant vector pLXSN-VEGF: According to compatible restriction sites in pcDNA 3.1-VEGF and pLXSN, *EcoR*I and *Xho*I were to digest the plasmid pcDNA 3.1-VEGF and pLXSN. Agarose electrophoresis and gel extraction were performed to acquire VEGF cDNA fragment and digeste pLXSN, then T4 DNA ligase was used to connect cDNA and plasmid (ratio: 3:1). After 16 h reaction, ligation mixture was transformed into *E. coli* JM109, and 6 separate colonies were harvested next day. After propagation plasmids were extracted to perform restriction analysis to identify the construction.

2.2.2 Packaging virus and determining viral titer: The procedures were performed following the User Manual[6]. Briefly, 5×10^5 PA317 packaging cells were seeded on 35 mm culture dish and cultured in 37℃, 5% CO_2 incubator for 18 h. When cells grew to 75% confluence, removed the culture, rinsed the cells with serum-free medium, used Lipofectamin to mediate recombinant plasmid transfecting pA317 packaging cells: 2 μg plasmid and 10 μL Lipofectamin diluted to 100 μL with serum-free and antibody-free DMEM, and slightly mixed together. The mixture were tiled to the layer of pA317 cells, placed in 37℃, 5% CO_2 incubator for 15 h. Next added 1 mL DMEM containing 20% FBS into it, and next day replaced the culture with normal solution. After 48 h, began screening using culture solution containing 800 mg/L G418. This process lasted for 3 weeks. Placed NIH 3T3 cells in 6-well plates at a density of $0.5-1\times10^5$ cells per well. Add 2 mL medium per well. Prepare 20 mL of complete medium and added 60 μl of 4 mg/ml polybrene. Collect virus-containing medium from packaging cells. Prepare six 10-fold serial dilutions. Infected NIH 3T3 cells by adding 1 mL of the diluted virus medium to the wells. Final polybrene concentration will be 4 μg/ml. The viral titer corresponded to the number of colonies present at the highest dilution that contains colonies, multiplied by the dilution factor.

2.2.3 Rat embryonic NSCs isolating culture and identification: NSCs were isolated from embryonic mice (day 16 - 18) essentially as described[7]. Briefly, embryonic brain was cleared of meninges, then separated the whole brain, rinsed with HBSS solution and sheared to small tissue pieces, digested with 0.125% pancreatic enzyme at 37 ℃ water bath. Filtered and centrifuged, removed supernatant, cultured by DMEM/F12 containing 2% B27, EGF (20 ng/ml), bFGF (20 ng/ml). Changed the liquid every 3 to 4 days and proliferated every 7 days. Limiting dilution assay was used to prepare monoclonal cell, immunocytochemistry was used to identify Nestin expression in NSCs. DMEM/F12 with 5% FBS was to induce NSCs differentiation. Immunocytochemistry was used to identify MAP2, GFAP expression in differentiated cells.

2.2.4 Infecting NSCs: Secondary generation neurospheres were separated into two sets: one was added with virus, and the other was treated with an insert-free virus as control cells. All were infected at a multiplicity of infection and cultured for 20 h. Transduced cells were cloned in G418 (200 μg/ml) and expanded for analysis of VEGF transgene expression. Immunocytochemistry was used to identify Nestin expression in transgenic NSCs; DMEM/F12 containing 5% FBS was used to induce NSCs differentiation and immunocytochemistry was used to identify MAP2, GFAP expression in differentiated cells.

2.2.5 RT-PCR: Total RNA was isolated from transgenic NSCs according to the protocol recommended by the manufacturer (TRI reagent; Sigma, USA), then a One-Step RT-PCR kit was used to amplify VEGF cDNA fragment. Samples were treated for 30 min at 56°C, then 5 min at 94°C and then 30 cycles of amplification were performed as follows: 45 sec at 94°C, followed by 45 sec at the annealing temperature, and 90 sec at 72°C, with final extension at 72°C for 10 min. Forward primer (5′-GCAAATGGGCGGTAGGCGTG-3′) and the reverse primer (5′-ATA GGA TCC TCA CCG CCT CGGCTT-3′) amplified a 570-bp pLXSN: VEGF specific gene transcript. β-actin transcripts were amplified from each RNA sample with β-actin-specific primers (upstream: 5′- TACAA CCTCC TTGCA GCTCC-3′, downstream: 5′-GGATC TTCAT GAGGT AGTCA GTC-3′) used as an internal control (620 bp). The RT-PCR amplification products were gel electrophoresed, stained with ethidium bromide.

2.2.6 Immunocytochemistry: Transgenic NSCs were fixed in 4% formaldehyde and the membrane was ruptured with 0.1% Triton for 10 min. After blocking cells for 30 min in diluent containing 10% goat serum, samples were incubated with primary antibodies overnight at 4°C. After rewarmed and rinsed biotinylated goat anti-mouse IgG was added, rinsed with PBS, added with SABC, rinsed with PBS again and stained with DAB. After stained with hematoxylin and alcoholic dehydrating and mounting, the samples were observed by light microscope. NSCs treated with an insert-free virus were as control cells.

2.2.7 Enzyme-Linked Immunosorbent Assay (ELISA): The supernatant of transgenic NSCs was collected and ELISA was performed as User Mannal described. According to the experimental data, curve of VEGF secretion state at different time was drawn.

3 Results

3.1 Restriction analysis and sequencing of pLXSN-VEGF

pLXSN-VEGF was digested by *EcoR*I and *Xho*I, then Agarose electrophoresis was performed. The results showed a 570 bp product and a 6,000 bp product, corresponding to VEGF and pLXSN. Linker region and VEGF cDNA were sequenced. There were no mutations generated from the plasmid construction, and the VEGF gene remained in frame.

3.2 Viral titer

After infected by retroviral, NIH 3T3 cells formed resistant cell colony after 3 weeks under the selective medium. The average viral titer was 6.5×10^5 cfu/ml, the highest was 1.8×10^6 cfu/ml.

3.3 Rat embryonic NSCs identification

Cells obtained from embryonic rats grew in suspension in DMEM/F12 and showed propagation manifest (Figure 1). The third generation of NSCs were fixed 2 hours after adherence, and most expressed the neuron specific protein Nestin. After differentiation the astrocyte marker GFAP and neuron marker MAP2 were expressed.

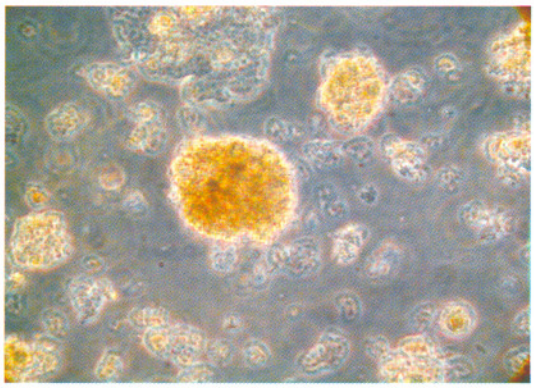

Figure 1. Neural stem cell globe (×400)

3.4 Infection of NSCs

Transgenic NSCs still remained in undifferentiated state. Immunocytochemistry results showed that the transgenic NSCs expressed the neuron specific protein Nestin (Figure 2). 7 days after adherence, most cell colonies differentiated into neurons, astrocytes and oligodendrocyte of typical shape. Immunocytochemistry showed that there were MAP-2 positive expression and GFAP positive cells (Figures 3,4).

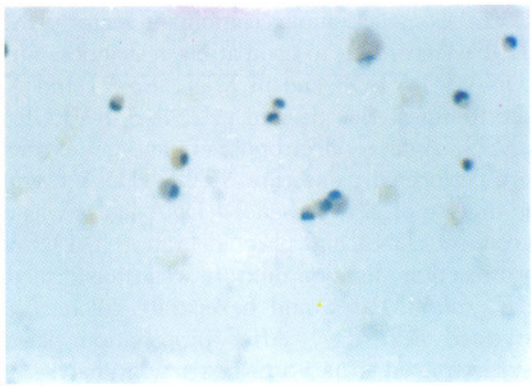

Figure 2. The positive expression of nestin antigen (×400)

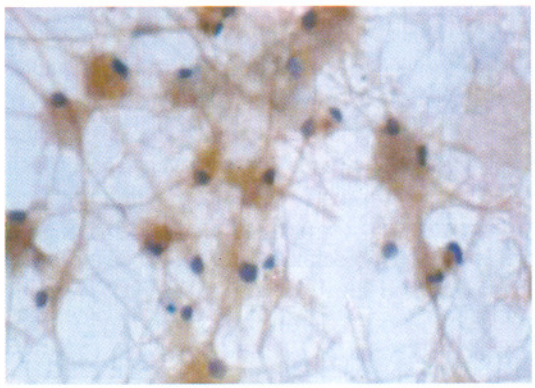

Figure 3. Identification of the expression of MAP-2 in glial cells by immunohistochemistry method (×400)

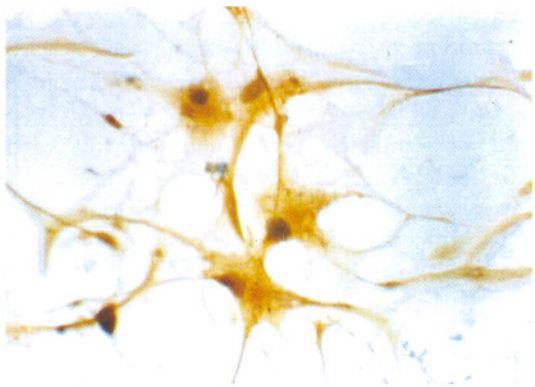

Figure 4. Identification of the expression of GFAP in neurons by immunohistochemistry method (×400)

3.5 RT-PCR and immunocytochemistry

RT-PCR studies verified transgenic VEGF mRNA in transduced clones (Figure 5), immunocytochemistry showed that the transgenic NSCs expressed VEGF protein (Figure 6), and NSCs treated with an insert-free virus were VEGF negtive.

3.6 ELISA

After transfection, concentration of VEGF in

supernatant gradually increased, and achieved peak in 6－8 days, then gradually descended until stabilization (Figure 7). The expression was maintained for at least 1 month (the last time point tested). After freeze-thaw, the transgenic NSCs were still able to stably secrete VEGF. In contrast, normal NSCs kept to secrete VEGF at a lower level.

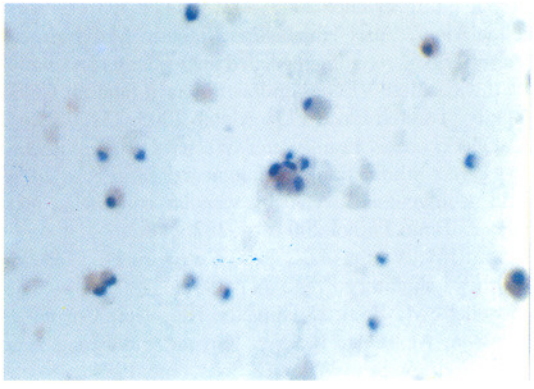

Figure 5. Identification of the expression of VEGF gene in transgenic neural stem cells by immunohistochemistry (×400)

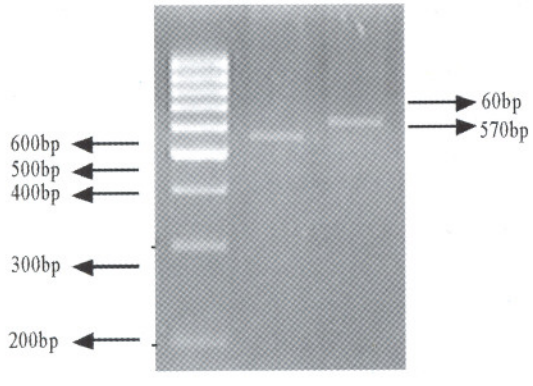

Figure 6. RT-PCR result of transgenic neural stem cells.
1: Maker 2: VEGF 3: β-actin

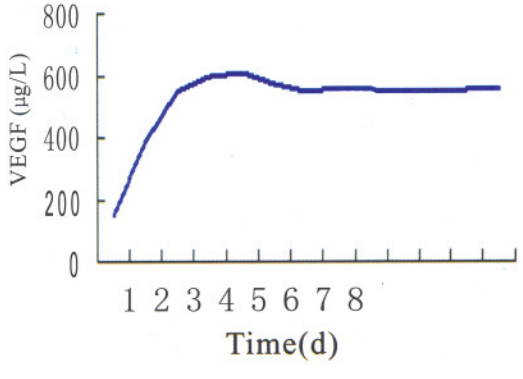

Figure 7. VEGF expression curve determined by ELISA

4 Discussion

NSCs are considered a heterogeneous population of mitotically active, self-renewing, multipotent, immature progenitor cells[4], making them uniquely situated to restore nervous system and therapy of nervous degenerating diseases. They may also present an ideal route for cell-mediated gene therapy as well as offer new possibilities for the replacement of neurons lost by injury or disease[8].

VEGF is a potent and specific mitogen for vascular endothelial cells. Anne has reported that hypoxia induced VEGF expression in clonally-derived adult rat neural stem cells *in vitro*, and low dosage of VEGF (2.4 ng/d) could stimulate adult neurogenesis *in vivo*[5]. He observed that VEGF could reduce neural stem cells apoptosis without altering its proliferation. This suggests that VEGF has survival promoting effect in neural progenitor cells[9]. We propose that VEGF acts as a trophic factor for neural stem cells *in vitro* and for sustained neurogenesis in the adult nervous system. The long-term and stable expression of VEGF by NSCs through retrovirus mediated gene transfer would enhance the concentration of VEGF maybe useful for the therapy of neurodegenerative diseases.

Retroviral gene transfer can efficiently introduce stable, heritable genetic material into the genome of any dividing cell type. pLXSN contains elements derived from Moloney murine leukemia virus (MoMuLV) and Moloney murine sarcoma virus (MoMuSV), and is designed for retroviral gene delivery and expression[10]. Upon transfection into a packaging cell line, pLXSN can transiently express, or integrate and stably express, a transcript containing viral packaging signal, the gene of interest, and a selectable marker. The 5′ viral LTR in this vector contains promoter/enhancer sequences that control expression of the gene of interest in the multiple cloning site. The SV40 early promoter (P_{SV40e}) controls the expression of the neomycin resistance gene (Neo^r), which allows antibiotic selection in eukaryotic cells. pLXSN also includes the Col E1 origin of replication and *E. coli* Amp^r gene for propagation and antibiotic selection in bacteria.

pLXSN does not contain the structural genes (*gag*, *pol*, and *env*) necessary for particle formation and replication. However, these genes are stably integrated into pA317 packaging cells. Subsequent introduction of pLXSN, containing the extended viral packaging signal (*psi*), transcription and processing elements, and the gene of interest

produces high-titer, replication incompetent infectious virus. That is, these retroviral particles can infect target cells and transmit the gene of interest (which is cloned between the viral LTR sequences), but cannot replicate within these cells since the cells lack the viral structural genes. Transforming NIH3T3 cells showed the average viral titer was 6.5×10^5 cfu/ml, in which the highest was 1.8×10^6 cfu/ml.

After transfection of NSCs, we detected the VEGF concentration every 3 days. The data showed peak of VEGF concentration was on the 6th – 8th day, then descended slightly, and reached its stabilization. After freeze-thaw, the transgenic NSCs were still able to stably secrete VEGF.

In this experiment, VEGF gene stably expressed in NSCs and the transfected NSCs still maintained its multipotency and self-renewal. These results hold promise for the use of genetically manipulated stem cells for CNS therapies.

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Potential and Mechanism of Peroxynitrite to Mediate Heme Degradation

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Abstract: The reaction of heme with peroxynitrite was studied. The results showed that peroxynitrite was able to degrade heme in solution with optimum pH of 7.4 and resulted in the release of iron from heme. Degradation depended on the peroxynitrite concentration with perferryl reactive species such as sodium sulfide and peroxidase substrate (phenol), demonstrated that perferryl reactive species formation was required for heme degradation. It was inhibited by superoxide dismutase, implicating the involvement of $O_2 \cdot^-$ in the process of heme degradation. [Life Science Journal. 2006;3(2):55-60] (ISSN: 1097-8135).

Keywords: heme degradation; peroxynitrite; reaction mechanism

Abbreviations: BSA: bovine serum albumin; DTPA: diethylenetriaminepentaacetic acid; Hb: hemoglobin; metHb: methemoglobin; oxyHb: oxyhemoglobin; HSA: human serum albumin

1 Introduction

The most sizable and concentrated store of heme in the body resides is erythrocytes Hb. As long as heme is bound tightly to its hydrophobic pocket in globin, it mediates its normal physiological role in oxygen transport. However, in some pathological conditions or under oxidative stress, heme may be released and exert various noxious actions[1]. The normal red cell is endowed with efficient mechanisms to remedy these physiological deviations, but variant erythrocytes such as sickle and thalassemic cells are unable some times to do so, either because their Hb has a higher tendency to auto-oxidization or is less stable. Unstable metHb readily forms hemichromes which have a tendency to bind to the cell membrane and sometimes may release their heme. Normal Hb were also shown to release their heme to cell membranes and to liposomes made of aminophospholipids, although at a reduced extent compared with metHb or hemichromes. Heme has been shown to rapidly destabilize the bilayer structure, thus increasing its permeability to ions and leading to hemolysis[2], and to induce the peroxidation of the membrane lipids[3]. Its binding to membrane proteins diminishes their reduced thiol content and leads to cross-linking. Both latter processes are apparently due to the chronic effect of increased membrane heme. Hence, efficient mechanisms must exist to prevent the build-up of heme in membranes. Since heme is able to translocate across membranes[4], it's fate and membrane concentration are determined by the presence of various ligands, such as serum's albumin and hemopexin[5]. Altogether, it is presumed that the concentration of free heme in the membrane is physiologically kept at low (micromolar) levels[6]. In conditions that favor higher membrane heme association, the concentrations of non-heme iron also increase in the membrane[7]. The origin of this iron is not well established, but it has been suggested that it could result from the destruction of heme by organic and lipid peroxides and by H_2O_2.

Recently, peroxynitrite chemistry is of considerable interest as a result of the increasing evidence for the role of peroxynitrite in the development of oxidative damage in various pathologies[8]. Peroxynitrite is formed *in vivo* from the diffusion-controlled reaction between the nitrogen monoxide and superoxide radicals[9]. Although these precursors are relatively unreactive, peroxynitrite is a powerful oxidizing and nitrating agent that can react with biological substances such as protein[10], nucleic acid[11], lipids[12] and carbonate[13] in cells and tissues. Peroxynitrite is relatively long-lived, which allows it to reach critical targets in the cell. Our

previous studies have shown peroxynitrite can promote the conversion of oxyHb to metHb and mediate damage to porphyrin ring of Hb active center, subsequent instability and heme loss from the protein[14]. But the studies on the direct reaction between peroxynitrite and free heme have not been reported yet.

In the present work, the interactions between free heme and peroxynitrite were investigated. The experimental results showed that co-incubation of these compounds led to the destruction of heme and the generation of oxidative radicals. These results indicated that peroxynitrite interacted with heme to produce an oxidative stress and increased the iron content.

2 Materials and Methods

2.1 Chemicals

Materials were obtained from the following sources: diethylenetriaminepentaacetic acid (DTPA), heme, catalase, superoxide dismutase, bovine serum albumin (BSA), human serum albumin (HSA), EDTA, Ferrozine (3-(2-pyridyl)-5, 6-bis(4-phenylsulfonic acid)-1, 2, 4-triazine) and neocuproine (2, 9-dimethyl-1, 10-phenanthroline) from Sigma. All other chemicals were of the best available grade and have been used without further purification, unless stated otherwise, and doubly distilled water was used throughout.

2.2 Preparation for peroxynitrite

Peroxynitrite were synthesized by reaction of 0.6 mol/L nitrite with 0.7 mol/L H_2O_2 at pH 13 and characterized according to the method reported previously[14]. Excess H_2O_2 was removed by passage through a column of manganese dioxide. The desired concentration of peroxynitrite was prepared daily by the dilution of the stock solution in aqueous 0.5−1.0% NaOH and this solution was kept in an ice bath (less than 10% of peroxynitrite had decomposed over 8 h). Peroxynitrite was stored at −20 ℃ and the concentration of peroxynitrite was determined spectrally in 0.1 mol/L NaOH at 302 nm ($\varepsilon_{302} = 1,670\ mol^{-1} \cdot L \cdot cm^{-1}$) immediately prior to each experiment[15]. After 3 − 5 days at room temperature the peroxynitrite had completely decomposed; this solution was called "decomposed peroxynitrite" and has been used in blank experiments.

2.3 Investigation of the destruction of the heme molecule by peroxynitrite

Heme was prepared fresh at the beginning of each experiment as a stock solution of 1 mmol/L in of 20 mmol/L. Fresh peroxynitrite solution was prepared as a stock of in 0.1 mol/L NaOH. First, DTPA was added (1 mmol/L final concentration) to the PBS buffer pre-warmed to 25°C. Then heme was added to the desired final concentration. Finally, different amount of peroxynitrite were added to give a serials of peroxynitrite concentration. The spectral changes between 300 nm and 800 nm were measured immediately after gentle mixing in a TU-1800pc UV-vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd, China) and thereafter at 100-s intervals using 1 cm light path quartz cuvette. All pH values were measured with a pH S-301 digital ion meter.

The absorbance at 390 nm was recorded after 30 min reaction. The pH dependence of heme degradation by peroxynitrite was measured as described above, in PBS adjusted to the desired pH. The pH was determined at the beginning and at the end of the reaction and was found to be constant. It was ascertained that heme did not precipitate out of solution during the measurement.

The effect of sodium sulfide, phenol, DTPA, EDTA, benzoic acid, BSA, HSA, superoxide dimutase and catalase on the heme degradation was also studied. These agents were added to heme 15min before initiating the reation with peroxynitrite.

2.4 Iron release from the heme molecule during the degradation reaction

A reaction mixture of the heme decomposing system was prepared in a final volume of 3 ml at 25 ℃. The heme concentration was 10 μmol/L, and different amounts of peroxynitrite were added. DTPA was not added to this reaction because it interfered with iron determination. Free iron was measured by the Ferrozine method[16]. Briefly, a 0.45 ml sample taken from the reaction mixture was mixed with 50 ml of 100% (*w*/*V*) trichloroacetic acid. Then, 0.5 ml of 0.02% ascorbic acid in 0.1 N HCl was added, the system was incubated for 5 min at room temperature, and 0.4 ml of ammonium acetate (10%) and 0.1 ml of Ferrozine solution (75 mg of Ferrozine and 75 mg of neocuproine in 25 ml water) were added. After an additional incubation for 5 min at room temperature, the color developed was measured at 562 nm.

3 Results

3.1 Degradation of heme by peroxynitrite

Mixing of heme with peroxynitrite results in a rapid decline in peak absorbance, indicating the

that degradation succeeds through various intermediate products. The final product of this reaction was obtained after substantially longer incubations (>2 h). The chemical nature of the end product (s) has not been investigated.

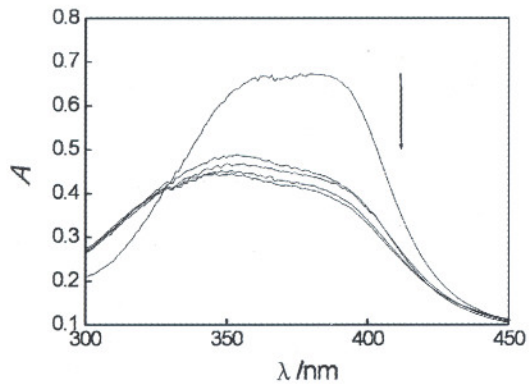

Figure 1. Heme degradation by peroxynitrite.

Heme (15 μmol/L) was mixed with 335 μmol/L peroxynitrite + 1 mmol/L DTPA in PBS, pH 7.4, and incubated at 25 ℃. Absorption spectra (300 − 450 nm) were taken at 120 sec intervals starting immediately after mixing.

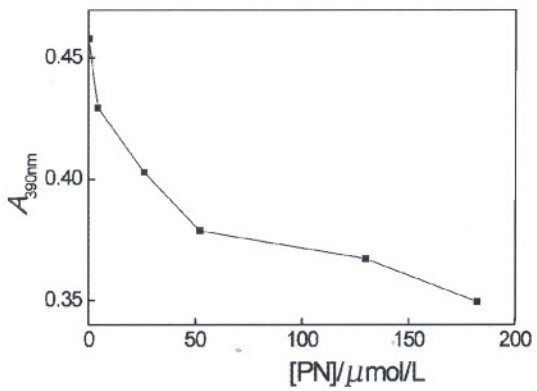

Figure 2. The dependence of heme degradation on peroxynitrite concentration.

Heme and peroxynitrite were mixed + 1 mmol/L DTPA at the desired concentrations in PBS, pH 7.4, at 25 °C, and heme degradation was monitored for 600 sec at 390 nm. [heme] = 10 μmol/L.

As shown in Figure 2, the rate of heme degradation was peroxynitrite-dependent, which are well consistent with those described for heme degradation by H_2O_2[17], but no further attempts were made to determine the precise molecular mechanism.

3.2 Effect of pH on peroxynitrite-mediated heme degradation

Heme degradation was pH-dependent in 0.1 mol/L PBS, peaking at pH 7.4 (Figure 3). The technique used for the assay of heme degradation precludes the possibility that the low rates observed at acid pH were due to precipitation of heme.

3.3 The liberation of iron from degraded heme

The decomposition of the heme molecule led to the liberation of the heme iron as determined by the Ferrozine method[16] (Figure 4). The release of heme iron was peroxynitrite-dependent.

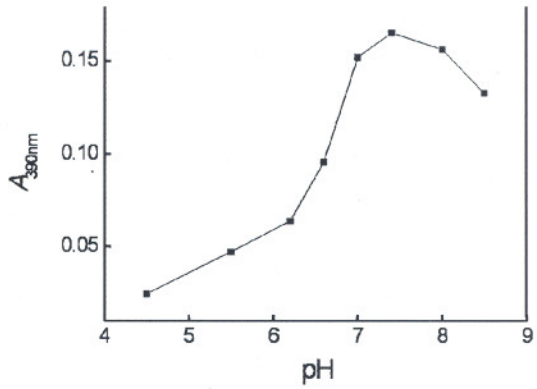

Figure 3. The dependence of heme degradation on pH.

Heme (10 μmol/L) was mixed with 250 μmol/L peroxynitrite and 1 mmol/L DTPA in PBS preset to the indicated pH. The heme degradation was determined by the decrease of the absorption at 390 nm.

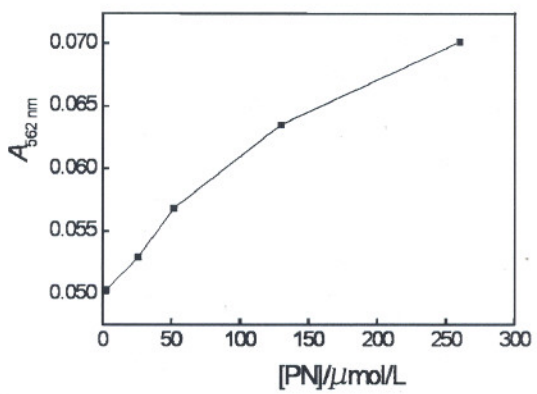

Figure 4. Iron releases from heme in the presence of peroxynitrite.

The reaction conditions were the same as described in Figure 2. The iron concentration was determined by the Ferrozine method.

3.4 Effect of various treatments on peroxynitrite-mediated heme degradation

The effect of prior addition of various reagents on the heme absorption was investigated. Reagents which reacted with ferryl reactive species were inhibited. Thus, sodium sulfide inhibited about 52% heme degradation. While phenol inhibited about 68% heme degradation. DMSO and benzonic acid, putative ·OH radical quencher, had no significant effect on the absorption. Two iron chelators, EDTA and DTPA, had no effect on the absorption.

This implies that ·OH radicals and free iron were not necessarily involved in the heme degradation. In spite of this fact, we included DTPA regularly in order to discard any nonspecific heavy metal-catalyzed peroxynitrite oxidation reactions.

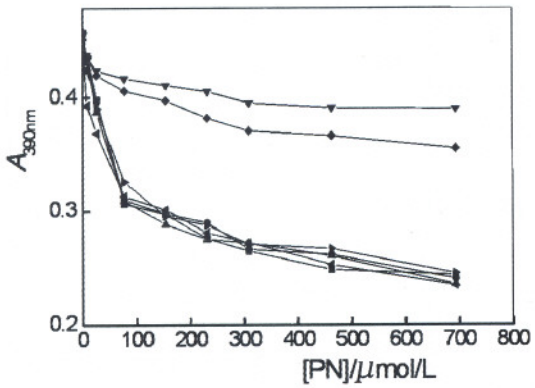

Figure 5. Effect of various treatments on the heme degradation.

The assay mixture contained 10 μmol/L and different amount of peroxynitrite in 0.1 mmol/L PBS (pH 7.4) in a total volume of 3 mL. The reaction was performed at pretreatment, (■) no treatment, (●) EDTA (0.1 mmol/L), (▲) DTPA (0.1 mmol/L), (▼) phenol (1 mmol/L), (◆)Na_2S (1 mmol/L), (▶) benzoic acid (1 mmol/L), (◀)DMSO (1 mmol/L). Absorption at 390 nm was recorded after reaction for 10 min.

Degradation also occurred when heme was bound nonspecifically to protein. Heme was complexed with defatted BSA and HSA in PBS by mixing the two compounds for 30 min at room temperature at a molar ratio of 70:1 (heme:albumin) and then subjected to the same spectrophotometric assay in the presence of peroxynitrite as done with free heme. The degradation of protein-bound heme was somewhat lower than that of free heme (Figure 6).

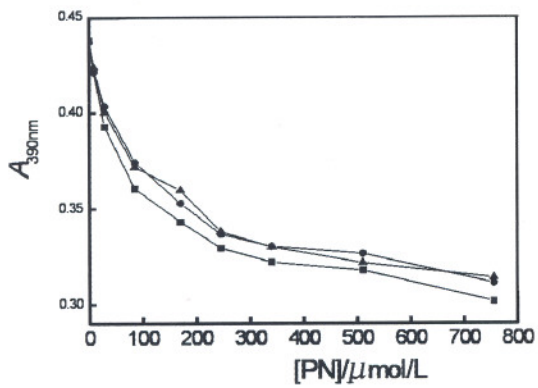

Figure 6. Effect of albumin on the heme degradation mediated by peroxynitrite.

The reaction conditions were the same as described in Figure 5. The reaction was performed at pretreatment, (■) no treatment, (●) BSA, (▲) HSA. Absorption at 390 nm as recorded after reaction 10 min.

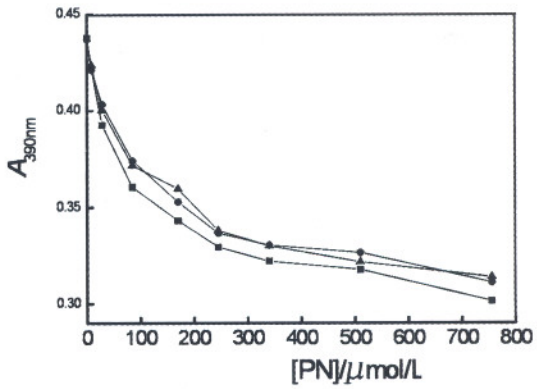

Figure 7. Effect of CAT and SOD on the heme degradation.

The reaction conditions were the same as described in Figure 5. The reaction was performed at pretreatment, (■) no treatment, (●) SOD (40mg/mL), (▲) CAT (128 U/mL). Absorption at 390 nm was recorded after reaction 10 min.

Superoxide dismutase (40 mg/mL) inhibited heme degradation by 46%. While catalase (128 U/mL) had no significant influence on the heme degradation (Figure 7).

4 Discussions

Structural changes in the globin molecule may lead to serious modifications of the hydrophobic heme binding pocket[18], ensuing in the loss of heme to other cell components, mainly to the membrane compartment of the red cell. High heme levels were detected in the membrane of abnormal red cell, such as b-thalassemic[19] and sickle cells[6,7]. In parallel, a significant phospholipid-bound fraction of non-heme iron was detected in the membranes of these cells[7,20]. Iron decompartmentalization was recently suggested to be an important feature of abnormal red cell and an important cause for cell lysis[21]. Knowledge about the mechanism which leads to the release of free iron detected in abnormal red cell is scarce. Iron can be released from hemoglobin or heme molecules by hydroperoxides[21]. The parallel increase of membrane heme and non-heme iron concentrations suggests that iron derives from heme[7], but the mechanism responsible for this phenomenon, or the elimination of excess intracellular heme altogether, remains an enigma.

Our previous studies have shown that heme moiety is partially degraded during the reaction of peroxynitrite with oxyHb. We have now established the reactions responsible for free heme degradation. The interaction between hemeproteins and peroxynitrite has been known for some time[22], but here we show directly, and for the first time,

that the interaction of free heme and peroxynitrite leads to a destruction of the tetrapyrrole ring of heme. This is evidenced by the unique absorbance of the final product of the reaction, the peroxynitrite dependence of this process (Figure 2), and the release of iron from the destroyed tetrapyrrole ring (Figure 4). We have observed that the degradation of heme is maximal at pH 7.4 and almost undetectable in PBS at pH 5 (Figure 3). The conclusion of these experiments is that peroxynitrite-dependent heme degradation occurs at physiological conditions, even when heme is bound nonspecifically to protein.

The decomposition of heme by peroxynitrite was not affected by traces of free iron which usually contaminate various chemicals, since no effect was seen in the presence of the iron chelators DTPA and EDTA, suggesting that iron released due to heme decomposition does not participate in the destruction of the tetrapyrrole ring.

At the present time, one can only speculate about the mechanism of peroxynitrite-dependent heme degradation. The oxidation of the various substrates by peroxynitrite ($OONO^-$/ONOOH) can take place via multiple pathways[15] (Scheme 1): (i) Peroxynitrite may directly oxidize the substrates. (ii) Peroxynitrite may decompose firstly into highly reactive species ($\cdot OH$, $\cdot NO_2$), which subsequently oxidizes the substrate or hydroxylates and nitrates aromatic compound. In the present study, the possible mechanism of the oxidation of heme by peroxynitrite was studied. Firstly, DMSO and benzoic acid (1 mmol/L), the specific scavenger for $\cdot OH$, was introduced to the reaction mixture before the addition of peroxynitrite, and the absorption of the system was almost unchanged compared with that in the absence of DMSO or benzoic acid, proving that $\cdot OH$ does not mediate the absorbance decrease of the system. Second, when SOD (40 mg/mL), a specific scavenger for $O_2^{\cdot -}$, was added to the reaction system, we found that the absorbance decrease of the reaction mixture was inhibited 46%, indicating that $O_2^{\cdot -}$, contribute to the absorbance increase of the system. As mentioned above, peroxynitrite predominantly reacted with hemoglobin in peroxynitrite anion form.

$$ONOO^- + H^+ \rightleftharpoons ONOOH \quad (1)$$

$$ONOO^- \rightleftharpoons \cdot NO + O_2^{\cdot -} \quad (2)$$

$$ONOOH \rightleftharpoons \cdot OH + \cdot NO_2^- \quad (3)$$

Scheme 1

Ferrylheme, is an intermediate of reaction. The requirement for ferrylheme is indicated by the dramatic inhibition of heme degradation when ferrylheme reacts with peroxidase substrates phenol or sodium sulfide. Thus, the peroxidase substrates, phenol, which reduce ferrylheme to metheme, completely inhibit the heme degradation, and sodium sulfide, which reacts with ferrylheme to produce sulfheme, results in an inhibition of heme degradation. These results indicated that heme degradation involved reactions of ferrylheme and was not the primary reaction of peroxynitrite with heme.

In conclusion, the present investigation indicates that heme is decomposed by peroxynitrite, either when heme is free or bound to proteins. During this process, heme is oxidized, oxidative radicals are produced, and iron is released. Further research is needed in order to verify the nature of the degradation products of the tetrapyrrole ring, and the types of the free radicals generated during peroxynitrite-dependent heme degradation. However, the elucidation of this mechanism and the identification of the reaction products are not pertinent to the present work which seeks to investigate the ability of peroxynitrite mediates heme degradation and effect of various pretreatments on heme degradation mediated by peroxynitrite.

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A New Characterized Y-STR and Its Allele Frequencies Distribution in 8 Chinese Populations

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Abstract: Y chromosome genomic DNA was used to search for new Y-specific short tandem repeats (Y-STRs) and a new Y-STR, DYS708, has been characterized. In 8 different Chinese populations, including Guangdong Han, Henan Han, Bai, Tibetan, Uygur, Tujia, Mongolia and Zhuang, 9 successive alleles have been found by PCR-based method. The sequences of different alleles showed that this locus included four blocks of AGAT and two blocks of AGAC and that length variations between existed not only in the largest block of AGAT, but also in the larger block of AGAC repeats. The name of each allele was the sum of the repeats number of these six repeats blocks ranging from 22 to 30 according to ISFG. The gene diversity was ranged between 0.7659 in Uygur and 0.6327 in Bai. Based on Nei's genetic distance, the genetic tree of these 8 populations was constructed. It was showed in the tree that Guangdong Han was closer to Bai than to Henan Han indicating gene flow between Guangdong Han and local minority, and Tujia was closer to Henan Han than to Guangdong Han implying that Tujia might come from northern part of China. These results agreed with the conclusion based on other genetic markers. All of the results indicated that DYS708 is a useful genetic marker and could be applied to human evolution study and forensic science. [Life Science Journal. 2006;3(2):61-65] (ISSN: 1097-8135).

Keywords: Y chromosome; short tandem repeats; gene diversity; human evolution; personal identification

Abbreviations: ISFG: the International Society for Forensic Genetics; NRY: non-recombining region of Y chromosome; PCR: polymerase chain reaction; Y-STRs: short tandem repeats on Y chromosome

1 Introduction

Human Y-STRs are tandemly repeated arrays of two to six base-pair units on NRY (also know as MSY, man specific region of Y chromosome). As genetic markers, just as its counterpart on the rest of chromosomes, Y-STRs are abundance, high degree of polymorphism and ease of scoring. It exists only in male and is transmitted only from father to son as a haplotype making it a useful tool in diverse fields. In forensic science[1], to identify a male suspect, there is no need to separate the male part of sample mixtures derived from male and female. Y-STRs also can be used to identify male lineage[2], which is difficult to use autosomal STRs. In anthropology Y-STRs can be used to trace male evolution[3-5], just as its counterpart, mitochondria DNA, in female evolution. Some diseases have been found related to some haplotypes defined by Y-STRs[6] and other markers. Although many Y-STR have been reported, the additional well-characterized Y-STR is still necessary in order to increase the discriminating power and the chance of exclusion in forensic science and to get high-resolution haplotypes of Y chromosome for its full use in human evolution and human genetics. The allele distribution of Y-STR is subject to natural selection, selective sweep, genetic draft, bottleneck events, population expansion and migration, so different population usually has different allele frequency distribution. This distribution is the basis of its use in many fields especially in forensic science. Here we described a new Y-STR, DYS708, which was identified from genome sequence of NRY. We also investigated its allele distribution in eight Chinese populations and constructed the genetic tree based on these genetic data.

2 Materials and Methods

2.1 Blood samples and DNA extractions

Blood samples of Henan Han (95 individuals) were collected in Henan province of China, while that of Guangdong Han (175 individuals) in Guangdong province of China, Tujia (60 individuals) in Hunan province of China, Zhuang (31 individuals) in Guangxi Zhuang Autonomous Region of China, Uygur (104 individuals) in Xinjiang Autonomous Region of China, Mongolia (49 individuals) in Inner Mongolia Autonomous Region of China, Tibetan (52 individuals) and Bai (138 individ-

uals) in Yunnan province of China. The ethnic group to which the blood donor belongs was determined according to the self claim of the blood donor. DNA was extracted using the Chelex 100 and proteinase K protocol[7].

2.2 Identification of novel Y-STR and PCR primer

Y-chromosomal DNA sequence data were obtained from GenBank (AC011298). Sequences were downloaded in FASTA format and used as input for the program Tandem Repeats Finder[8]. A Microsatellite with a 4-bp motif repeating 11 times was chosen from the output of this program as our research object. Primers were designed using Primer3 software. Unlabelled primers were synthesised by SBSBio Company (Beijing, China).

2.3 Amplification conditions and analysis of PCR products

The PCR was performed in a 20 μl final volume containing 1 × Taq™ buffer (10 mmol/L Tris-HCl, pH 8.3, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl), 200 μmol/L dNTPs, 30 – 50 ng DNA, 1 U Taq™ (TaKaRa Biotechnology Co., Ltd, Dalian, Liaoning, China). Primer sequences were: left primer AGTGTATCCGCCATGGTAGC, right primer CTGCATTTTGGTACCCCATA. In the TouchDown PCR protocol the DNA was initially denatured at 94 ℃ for 2 min. This was followed by 15 cycles starting at 94 ℃ for 0.5 min, 63 ℃ for 40 sec and 72 ℃ for 45 sec. The annealing temperature was decreased by 0.5 ℃ in each cycle. It was then followed by 20 cycles at 94 ℃ for 0.5 min, 55 ℃ for 40 sec and 72 ℃ for 45 sec. After a final extension step at 72 ℃ for 5 min the samples were kept at 4 ℃ until electrophoresis.

The amplification products (1 μl) were electrophoresized on 6% polyacrylamide gels (PAG) in 1× TBE buffer and the DNA bands were detected by silver stain[9]. The DNA bands at different length were eluted from gel and reamplified with the conditions previously described. The reamplified products were sequenced by BIOASIA Company.

2.4 Typing and nomenclature

Allele assignment of amplified respective DNA fragments was performed by side-to-side comparison with the allelic ladder consisting of sequenced alleles. The suggested repeat nomenclature of alleles follows the guidelines of the DNA commission of the International Society for Forensic Genetics (ISFG)[10]. Two female samples were included as negative controls.

2.5 Statistical analysis

Gene frequencies of alleles were obtained by simple gene counting. Gene diversity and standard errors were calculated following Nei[11]. MEGA version 3.0[12] was used to construct genetic tree (neighbor-joining method[13]) based on D (genetic distance) and D equal to $-\ln(J_{XY}/\sqrt{J_X J_Y})$ where $J_{XY}=\sum x_i y_i$, $J_X=\sum x_i^2$ and $J_Y=\sum y_i^2$. x_i and y_i are the frequencies of ith allele in population X and population Y respectively[14].

3 Results

3.1 The repeat structure and nomenclature of alleles

DYS708 (dbSTS-Id: 340593; GenBank-Accn: BV209666) primers amplified a DNA fragment specifically in male and no band was found in female. The amplified products have a sequence structure of AGTGTATCCGCCATGGTAGCATA ATAGAAATTTTATGAGTGGGAGAAATGGAT GACAGTAAAATGAAAACATTGCAATGTGTA TACTCAGAAACAAGGA (AGAT)mGAT(AGAT)n(AGAC)o(AGAT)p(AGAC)q(AGAT)r AGAATATATTATGGGGTACCAAAATGCAG (for the reference sequence in genbank, AC011289, $m=3$, $n=3$, $o=1$, $p=11$, $q=8$ and $r=2$). We have found 9 different alleles and at least one present sample from each allele was sequenced. There were no difference in m, n, o and r between the sequenced alleles, but p could be 6 to 13 and q could be 7, 8 or 9. The structures of sequenced alleles were summarized in Table 1. The suggested repeats nomenclature of alleles was the sum of m, n, o, p, q and r according to guidelines of the DNA Commission of the International Society for Forensic Genetics (ISFG).

3.2 Allele frequency distribution and gene diversity (GD)

In total 9 alleles have been found. 8 out of 9 alleles have been found in Henan Han population and only 4 alleles were found in each of Mongolia and Zhuang populations. The gene diversity ranged between 0.7659 (Uygur) and 0.6327 (Bai). The allele frequency and gene diversity were showed in Table 2.

Table 1. The structures of repeat blocks of DYS708 alleles

Allele (bp)	Repeat motif
23 (223)	99bp(AGAT)3GAT(AGAT)3(AGAC)1(AGAT)6(AGAC)8(AGAT)229bp
24 (227)	99bp(AGAT)3GAT(AGAT)3(AGAC)1(AGAT)7(AGAC)8(AGAT)229bp
25 (231)	99bp(AGAT)3GAT(AGAT)3(AGAC)1(AGAT)8(AGAC)8(AGAT)229bp
	99bp(AGAT)3GAT(AGAT)3(AGAC)1(AGAT)9(AGAC)7(AGAT)229bp
26 (235)	99bp(AGAT)3GAT(AGAT)3(AGAC)1(AGAT)9(AGAC)8(AGAT)229bp
27 (239)	99bp(AGAT)3GAT(AGAT)3(AGAC)1(AGAT)10(AGAC)8(AGAT)229bp
	99bp(AGAT)3GAT(AGAT)3(AGAC)1(AGAT)9(AGAC)9(AGAT)229bp
28 (243)	99bp(AGAT)3GAT(AGAT)3(AGAC)1(AGAT)11(AGAC)8(AGAT)229bp
29 (247)	99bp(AGAT)3GAT(AGAT)3(AGAC)1(AGAT)12(AGAC)8(AGAT)229bp
30 (251)	99bp(AGAT)3GAT(AGAT)3(AGAC)1(AGAT)13(AGAC)8(AGAT)229bp
31 (254)	99bp(AGAT)3GAT(AGAT)3(AGAC)1(AGAT)16(AGAC)8(AGAT)229bp

Table 2. The allele frequency and gene diversity in 8 Chinese populations

Allele (bp)	Guangdong Han	Henan Han	Bai	Tibetan	Uygur	Tujia	Mongolia	Zhuang
23 (223)	0	0.0105	0	0	0	0	0	0
24 (227)	0	0.0105	0.0072	0	0.0192	0	0	
25 (231)	0.0057	0.0105	0	0	0.0096	0	0	0
26 (235)	0.0743	0.0948	0.0072	0.0385	0.1154	0.0167	0.0816	0.0645
27 (239)	0.4172	0.3263	0.5290	0.3077	0.3365	0.4333	0.3878	0.129
28 (243)	0.2743	0.4	0.2464	0.2692	0.2596	0.4	0.3265	0.4194
29 (247)	0.2114	0.1369	0.1739	0.3654	0.2115	0.0667	0.2041	0.3871
30 (251)	0.0171	0.0105	0.029	0.0192	0.0481	0.0833	0	0
31 (255)	0	0	0.0072	0	0	0	0	0
	GD:0.7042	GD:0.7128	GD:0.6327	GD:0.7111	GD:0.7659	GD:0.6514	GD:0.7091	GD:0.6752
	SE:0.0123	SE:0.0179	SE:0.0208	SE:0.0151	SE:0.0127	SE:0.0238	SE:0.0191	SE:0.0301
	n:175	*n*:95	*n*:138	*n*:52	*n*:104	*n*:60	*n*:49	*n*:31

3.3 The genetic tree of populations

Based on Nei's genetic distance, the genetic tree of these 8 populations was constructed (Figure 1).

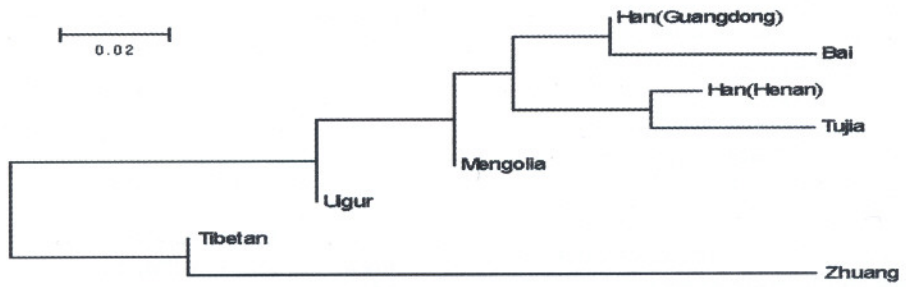

Figure 1. Genetic tree of 8 Chinese populations

4 Discussion

Human genome sequence data make it convenient to find novel polymorphic Y-STR just as did by Ayub[15] and Redd[16]. The euchromatin of Y chromosome is about 23 megabases (Mb) much of which contains sequences that shared with the X and other chromosomes or repeated elsewhere on itself[17], so the sequences on which Y-STR are searched for should be carefully selected in order to amplify Y specific sequence.

DYS708 could be classified as compound repeats since it consisted of two different motifs. The repeat region comprised four stretches of AGAT

and two stretches of AGAC. The variations of repeat number existed in the third block of AGAT repeats and the second block of AGAC repeats, but the former was more variable. Our nomenclature was based on the total number of repeats including six repeat blocks according to the recommendations of ISFG, not taking the sequence of GAT following the first block of AGAT into account, although GAT might be a AGAT motif having a deletion of A.

Compared with other reported Y-STRs, DYS708 was a highly polymorphic Y-STR locus, at least in the populations we have investigated. That Uygur population had highest gene diversity might be due to gene flow along the history of the Silk Road. Since Xinjiang Uygur Autonomous Region is close to Central Asia, the result that Uygur population had highest gene diversity agreed with the conclusion made by Wells that Central Asia is an important reservoir of gene diversity[18]. From the genetic tree, some conclusions could be come to. First, Guangdong Han was closer genetically to Bai than to Henan Han, implying gene flow between Han and local minority. Second, Tujia, inhabiting in southern part of China, was closer genetically to Henan Han (northern part of China) than to Guangdong Han (southern part of China), indicating that Tujia might come from northern part of China. All of these results agreed well with the conclusion based on other genetic markers (red blood group, HLA, red blood enzyme, etc)[19]. Contrary to the previous findings, Zhuang lay in a separate branch with Tibetan, the reasons for which was not clear and need further investigation.

Since Y chromosome is transmitted from father to son as a haplotype, its usefulness should be viewed as haplotype diversity. Although further investigations of its properties, such as its haplotype diversity with other Y chromosome genetic markers and its mutation rate, should be made before its full use in many fields, we still anticipated that this STR is a useful genetic marker and can be used in combination with other genetic markers in the studies on human evolution, the association study of some diseases and forensic science.

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The Most Comprehensive Truth Available to Living Humans

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Abstract: Human beings by their very nature make judgments of "true", "false" and "hypothetical" with reference to the various aspects and sectors of existence. Great numbers of these judgments conflict or contradict each other, with no possibility in sight whereby such discordance can be resolved. In view of this situation, the one most comprehensive truth accessible to humans is simply the inclusive, overarching metajudgment that all differential judgments have indeed been made. The conscious perception of this metajudgment marks in theoretical terms the determinate limit of the indeterminate extent of human knowledge of the total fabric of objective reality. [Life Science Journal. 2006;3(2):66-72] (ISSN: 1097-8135).

Keywords: life; human beings; judgments; true; false; hypothetical; comprehensive truth

One of the fundamental activities of living higher organisms, most profoundly in the case of human beings, is their inherent proclivity for making judgments of "true", "false", or "hypothetical" with reference both to themselves and to other sectors and aspects of existence. Within this framework the present paper will undertake to identify the single most comprehensive truth accessible to humans.

The reader may smile at the folly of attempting to cope with a topic of such magnitude. The ancient Chinese philosopher Zhuang Zi observed that life is limited, while knowledge (needed to perceive ultimate truth) is unlimited, so using the limited time of one's life to chase after unlimited knowledge (needed to perceive ultimate truth) is dangerous. Nonetheless, both Eastern and Western thinkers have continuously and tirelessly pondered the intriguing question of ultimate truth, and many persons continue to wonder how close humans can come to grasping one grand inclusive truth deriving from our own judging activity, and we feel the question still deserves the attempt to supply an answer. One possibility that might occur to anyone who considers the matter is that the apprehension by individuals of one maximally comprehensive truth would first require their mastery of the various established fields of learning. However, this approach is seen on the least reflection to be infeasible, for no person can assimilate during one lifetime the enormous range of specialized information now available-in the fields, say, of astrophysics, South Asian history, the etymology of African languages, electrical engineering, and a vast array of others. Moreover, even if such an assimilation by one person were possible, there is no warrant for supposing that accomplishment would yield one consolidated comprehensive truth rather than a vast clutter of findings. Nor can we suppose that experts from the many different fields of learning, assembled for the purpose of coalescing their widely separate esoteric findings into one grand coherent truth, would fare any better. In the following discussion, then, we will take a more promising approach to disclosing the one maximally comprehensive truth actually obtainable by living humans.

We would like first to recall the common assumption of a basic dichotomy in existence in terms of "what has happened in the past" and "what happens in the present". On close examination this distinction appears to be of little consequence, for any division between past and present shrinks to the vanishing point; on a strict view what happened some indefinitely divisible fraction of a second ago is as much a part of the past as what happened a billion years ago or an infinite time ago. Thus the past has included everything that has occurred up to the ever-receding present moment; but that moment is so abidingly elusive that it is not incredible to maintain that the past includes virtually all occurrence through all time. This is the meaning we have attached to the term "past" in the following discus-

sion.

More specifically, we conceive of the past as fundamentally twofold in character: thus the "past-as-actuality" comprising what has occurred, may be contrasted with the "past-as-understanding" comprising what has been apprehended through the agency of human judgment about that occurrence (Gottschalk, 1958). Or more precisely, since human judgments have themselves occurred, the past-as-actuality may be taken to include all that has occurred exclusive of such judgments. Moreover, due to an inherent characteristic of the human disposition for rendering judgments, the latter have been made on the differential basis of "true", "false", and "hypothetical". The past-as-actuality has been neither true, nor false, nor hypothetical; it has been, simply, "there"; truth, falsehood, and the quality of being hypothetical are exclusively properties of the human understanding of that actuality. Judgments of the past-as-understanding which to the satisfaction of certain persons have corresponded closely with given sectors of actuality in some sense of identifying, describing, or explaining them, have constituted for those persons particular truths. Judgments which to the satisfaction of given persons have not so corresponded, have constituted for those same persons particular falsehoods, while judgments concerning which, to the satisfaction of given persons the degree of correspondence has been uncertain, have for those persons constituted hypotheses. (We use the term "hypothesis" less in the technical scientific sense of an inference drawn from accumulated data in order tentatively to explain a general principle of nature, life, or society, than in the wider sense of any judgment regarded as being inconclusively true or false.)

The past-as-understanding in the broad sense here intended comprises judgments relating to the many sectors or aspects of actuality, and includes the judgments of everyday experience, as well as, on a more formal basis, the judgments of the natural and social sciences, the various technologies, philosophy, theology, etc. Thus the past-as-understanding has reference not only to judgments issuing from those disciplines of inquiry that provide knowledge is a positivistic sense, but also judgments of intuition and faith. From the vantage point of maximal generality judgments of "knowledge" and judgments of "faith" deserve equally the designations of true, false, or hypothetical; at this level of generality distinctions in kind between judgments relate simply to the sense of conclusiveness, whether as to truth or falsity, or to the sense of hypothetical possibility, involved in given judgments.

The past-as-understanding also includes the judgments of the field of learning known as "history". It might seem on first consideration that the latter subsumes the former. However, history as a discrete discipline of inquiry, the research methods of which constitute historiography, yields specialized judgments concerning political, social, economic, military, and other particular aspects of the past-as-actuality, and is thus simply one among other components of the past-as-understanding.

The pursuit of the past-as-understanding on the differential basis of true, false, and hypothetical judgments has often led to controversy; frequently different persons of equally good intention have rendered incompatible judgments in reference to the same sectors of actuality. Disagreements have been less extensive in some areas of understanding than others. For example, some judgments within the area of physical science (say with regard to the atomic weights within the periodic table of the chemical elements) or within the area of political history (say with regard to lines of succession within particular ruling dynasties) have enjoyed virtually universal concurrence among interested parties. At levels of greater complexity within such fields, however, judgments have differed from one interested party to another (say in reference the evolution of the physical universe, or to the causal configuration of major political episodes).

Within other areas of understanding, notably philosophy and theology, disagreement has remained widespread and stubborn (Hopfe, 1983; Levi, 1949). On the one hand, there has not been general agreement among people concerning the possible means to be employed in making judgments which correspond with actuality, or concerning the degree to which the filter of humans' own perceiving apparatus, or their proclivities for symbol formation (use of language), may distort such correspondence. In other words, in the exercise of judgment with reference to the nature of that part of actuality that is their own judging capacity, people have not achieved universal accord. Different persons have reached different conclusions in regard to the authenticity and efficacy of modes of judgment known as "empirical", "rational", "intuitive", and "mystical". Nor has there been general agreement concerning the essential nature or varieties of the perceived actuality itself. Different persons have been variously persuaded that their judgments have corresponded with sectors of actuality know as the "material", the "mental", the "conscious", the "subconscious", the "metaphysical" or the "divine". The past-as-understanding has been rich in disparate judgments concerning these matters,

without there having been discovered any criterion by appeal to which the differences could be resolved to the satisfaction of all concerned.

The opposition between some judgments of the past-as-understanding has been direct and complete, as in the case of the judgments "There is a god" and "There is no god". In other cases the contradiction has been partial, yet significant, for example between judgments both of which maintain there is some divine actuality, but one of which asserts polytheism, the other monotheism; or, granting the latter, one judgment which asserts pantheism, the other transcendence; or, granting the latter, one judgment which asserts providentialism, the other deism.

We submit that the past-as-understanding, as constituting a record of often discrepant and irreconcilable judgments, suggests that the one judgment which comprehends all others is that all other judgments have been made; that is, the one maximally comprehensive truth apprehensible by all parties to all conflicts of understanding consists in the recognition that there have been differential and often conflicting judgments rendered during the past. This concept is apprehensible, we should think, by any person who pauses to consider that his or her own judgments have in some cases been incurably at odds with the judgments of other persons in reference to the same reputed sectors of actuality – or who considers the extensive public record (to be confirmed in any library) of similar disagreements on the part of others – but who nonetheless yearns for some over-arching truth which accommodates all human judgments. The one maximally comprehensive truth as here conceived derives, then, not from the synthetic grasp in substantive terms of the judgments of all fields of learning, something which, as noted previously, is impossible of attainment. Rather the one maximally comprehensive truth is a "second order truth". While the less comprehensive judgments of the past-as-understanding have direct reference to given sectors of actuality, the one maximally comprehensive truth derives from a metajudgment in reference to the occurrence of those other judgments, and stands thus at a second remove from actuality.

The making of judgments on the differential basis of true and false involves the ancient Chinese principle of "*yin* and *yang*"; any judgments whatever that are asserted as "true" can have any significant meaning only in conjunction with the awareness that various other judgments are asserted as "false". If all judgments rendered by humans were automatically conceived of as true, such truth would be pointless. Thus the concept of one maximally comprehensive truth has been arrived at not only through recognizing that other judgments have been rendered, but just as crucially through ascertaining (on the basis of some degree of substantive understanding and through logical discernment) that those other judgments have been differential; that is, some of the other judgments have been true in contrast to some that have been false. And it follows that the rendering of judgments on the differential basis of true and false must hold as well for rendering the metajudgment of one comprehensive truth. On the metajudgmental level the judgment which contradicts the judgment of one maximally comprehensive truth is a form of solipsistic argument to the effect that no judgments whatever have been made beyond the consciousness of the individual offering the latter argument, a view which thoroughly violates our own intuition, and which we thereby judge to be false; and thus I hold the metajudgment of one maximally comprehensive truth to be itself differentially true.

The one maximally comprehensive truth is confirmed through the consideration that the past-as-understanding has ever been an expanding process. For example, humans did not discover and make judgments in reference to bacteria until the nineteenth century, or to viruses until the twentieth century – and so on in a vast number of equivalent cases. Moreover, while this ongoing process inspires of itself no anticipation that is will cease, it implies that human understanding has always remained limited to an indeterminate degree, in that humans have not been able at any point in the past to realize with what portion of the total fabric of actuality, from microcosm to macrocosm, their accumulated judgments have at that point corresponded. In other words, at all successive points in the past existence of humans their judgments have been, in substantive terms, collectively incomplete. But in that case the one complete (maximally comprehensive) truth available at any one of those successive points has resided in the metajudgment that a set of collectively incomplete other judgments has up to that point been rendered.

Some of the judgments subsumed within the one maximally comprehensive truth are of course both more comprehensive in their scope, as well as being more universally acknowledged as true, than are others. For example, within the area of scientific understanding the Principle of Conservation of Energy (the judgment that material energy as encountered by humans in the world can be neither created nor destroyed, but only converted form one form into another, while the total amount of energy remains constant) is both regarded as true by virtu-

ally all interested parties, and is universally applicable in its reference to actuality. Yet the Principle of Conservation of Energy (like many other comparable scientific judgments) is far from approaching the status of a maximally comprehensive truth, for it incorporates no judgments from many areas of profound and intimate concern to humans.

At the same time, among judgments that are relevant to such concerns, including those from the sectors of metaphysics and theology that purport to disclose, in terms even more comprehensive than scientific findings, the fundamental wherewithal or underlying dynamics of all actuality, are typically beset with abiding problems of mutual contradiction. For instance, what the universe cannot have been designed and determined is its operation by a transcendent Prime Mover, who has been perfect from all eternity, in the usual Christian sense, and at the same time be a universe which is coterminous with a dialectically evolving god in quest of its own identity, in the Hegelian sense. As contradictory world-formulae these judgments cannot alike be true, except as they are equally components of the one maximally comprehensive truth. At the same time the judgment, stemming from the philosophy of logical empiricism, that such formulae (being neither logical tautologies nor empirically verifiable) can be neither true nor false, nor even genuinely hypothetical, but only nonsensical (Ayer, 1952, 1991) is in the larger perspective of the one maximally comprehensive truth only one more contradictory judgment beside others (beside judgments which deny that supraempirical formulae are nonsensical).

As a further illustration of the concept of one maximally comprehensive truth, consider the sector of the past-as-understanding known as logic. Broadly speaking, work within the field of logic may be conceived of as an instance of humans standing over and against the actuality of themselves and attempting to understand themselves – to understand, in this case, the mechanism of that aspect of their judging capacity know as rational. More specifically, logic involves the establishment on an abstract level of the inferences of truth or falsity that certain kinds of judgments have for others. For example, within the sphere of deductive logic, premises of the type "All a is b" and "Some a is c", when taken together infer unavoidably the truth of the conclusion "Some c is b". As another example, the principle of contradiction (which is the essential factor at the core of the system of human differential judgments) can be expressed in the terse fashion of formal logic as "No sentence of the form 'p and not p' is true."

But while the specific findings of logic (an extensive and intricate array of findings far in excess of the simple examples given above) have been widely agreed upon by interested parties, if one presses for an answer as to what the deeper grounds of logical understanding are (Why do logicians think the way they do in arriving at their abstract principles?), one encounters a range of disagreement reminiscent of other sectors of the past-as-understanding. Some logicians have judged that logical understanding is empirically derived, that it reflects our experience in the world, that it echoes our consistent past observations of how the world actually works. Other logicians have judged that logical understanding, while indeed accurately reflecting the operation of the empirical world, is nonetheless derived on an *a priori* basis; it is an understanding purely rational or introspective in origin, which yet informs us about the nature of the external world. Still other logicians have maintained that logical understanding is a reflection of how the human mind itself compulsively functions; humans make the logical distinctions they do as a result of their minds being so constituted that they can make no other kinds of distinctions (this interpretation tends to transform philosophical logic into a branch of scientific psychology). Yet other logicians have concluded that logical understanding, rather than reflecting the necessary operations of human minds, or rather than being informative about further reaches of actuality, is based on verbal custom; logical insights are arbitrary conventions arising from the growth of language, and simply reflect the habitual meanings which humans have for convenience attached to words such as "and", "or", "all", "some" and "not" together with their syntactical relations (Barker, 1965).

Thus it appears that whatever the ground of logical understanding may be, the exercise of that understanding discloses that the several judgments concerning its basis are themselves mutually contradictory; they are judgments concerning which there are no available means of achieving a resolution irresistibly persuasive to all interested parties. We submit, however, that what must be persuasive to all interested parties is the occurrence of the debate among logicians in the exercise of their rival judgments. And the various sides to this debate, as well as to those of other debates from other areas of understanding, form components of the one maximally comprehensive truth to the effect that all such debates have occurred.

Questions of logic aside, within the past-as-understanding various judgments have been offered concerning the general type of judgments that de-

serve in turn the designation of "true" (such theories of truth being themselves, thus, metajudgments of a sort). The particular concept of truth that we ourselves find persuasive, as suggested by the foregoing discussion, is the "correspondence theory" according to which judgments are true if they agree with or parallel certain sectors or aspects of actuality. By contrast, the "coherence theory" of truth maintains that it is only judgments which are *consistent* with some wider system of judgments which deserve to be called true; such a system, within which particular constituent judgments imply the others, may be a broad metaphysical or theological scheme, or it may be the accumulated judgments of empirical science, or it may be a "definitional" system as in the case of pure logic (assuming that the ground of logical understanding is linguistic rather than empirical). On the other hand, the pragmatic theory of truth (in the one version of William James) holds that only those judgments which prove in the ongoing experience of humans to be satisfying deserve the designation of "true"; if a judgment is discovered to be personally or socially rewarding or useful in its consequences then it is true (James, 1988).

The circumstance that contradictory theories of truth have been advanced, and that we have admittedly derived the concept of one maximally comprehensive truth in part from one of these theories rather than another, does not, however, threaten the validity of the concept. For whatever rival judgments might be advanced by way of challenging an assumption from which the one maximally comprehensive truth has been derived are paradoxically embraced by the very truth they seek to challenge – the truth, namely, that all contradictory but less comprehensive judgments than itself have been rendered. Concerning the example in hand, insofar as rival theories of truth are themselves metajudgments, the one maximally comprehensive truth is a meta-metajudgment in reference to their occurrence.

The concept of one maximally comprehensive truth also derives in part from a certain view of time. As with other questions about the fundamental nature of existence, the question of time has engendered a long (and in this case especially tangled) record of contradictory judgments. The views of many persons have constituted some variation of one of the following notions. Some persons have inclined to the view that time is a self-subsistent entity or process "within" which substances change or move. Others have held that time has no existence apart from the motions which substances undergo, and is a construct devised by humans out of their perceptions of those motions. Still other persons have maintained that time is purely intuitive, or *a priori*, constituting one of the means whereby human beings impart order to their own experience. In the view of relativity physics time is one integral dimension of an empirically verifiable space-time continuum (Gale, 1967).

For our own part we accept the truth of the latter judgment, which I believe is consonant with statements made above to the effect that events have occurred in the past and that human judgments about those events have in the past been made. Relativity physics calls into question the concept of absolute time, through demonstrating that the temporal interval between events, or the simultaneity of different events, varies according to the spatial locations of different observers (such is the case, that is, at a "deeper" level of reality than can be perceived, or that need be considered, in the practical affairs of everyday life) (Barnett, 1950). But even relativity physics acknowledges an "absolute earlier" and an "absolute later"; for example, Einstein lived during an interval of time absolutely later than the interval during which Newton lived; any mother is born at a point in time absolutely earlier than the point at which her own child is born, and so one. And this concept is sufficient to support the one maximally comprehensive truth, which may be stated as follows: it is true that within a space-time continuum contradictory judgments in reference to time have been rendered, some of them absolutely earlier, or absolutely later than others (and for all practical purposes many of them concurrently). But while we thus utilize a particular judgment of the past-as-understanding in deriving the concept of one maximally comprehensive truth, the latter exceeds the particular judgment in question in the sense of also incorporating within its metajudgmental reach the occurrence of any or all contradictory judgments concerning the nature of time.

We further submit that what is commonly referred to as the "future" has for living humans no reality status apart from anticipations made up to and including the ever-receding present moment; there is no "future-as-actuality". Given anticipations have been, in the view of different persons, true, false, or hypothetical, as have been the later confirmations or revisions of those same anticipations, while all anticipatory judgments and their subsequent confirmations or revisions are subsumed within the one maximally comprehensive truth.

As mentioned above, there are no contradictions in the past-as-actuality – or, as some people might prefer to say, in "objective reality" – which

contains or exhibits no truth or falsehood, but simply exists; it is simply "there". Truth, falsehood, and the quality of being hypothetical are products solely of the human assessment of given aspects of the past-as-actuality, and the frequent conflicts within those differential judgments result from the limitations and imperfections of the finite human judging capacity. But while the one maximally comprehensive truth subsumes all differential judgments within the past-as-understanding, even the one maximally comprehensive truth is not *totally* comprehensive, because, as noted previously, that truth acquires the status of being itself differentially true only through reference to a contradictory metajudgment which it does not subsume. The one maximally comprehensive truth is only the *most* comprehensive truth available to finite human understanding. Hence, it might seem on first consideration that living humans could possess totally comprehensive truth only if they were omniscient. Yet, such an omniscient total truth, being in its own character completely non-contradictory, that is non-differential, could ironically be neither true nor false in the sense unavoidably employed by beings of merely finite understanding.

But if some of the judgments which are subsumed by the one maximally comprehensive truth, namely those of religious convictions, assert a correspondence with the actuality of one or another omniscient god, need such judgments vitiate for persons who make them the validity of the one maximally comprehensive truth? We think not, for the believers in an omniscient god do not claim that their own understanding is equal to that of god, but only that they apprehend some portion of god's understanding (say, for example, god's intentions toward humans). In other words, such human beliefs remain finite (and, taking into account all persons who hold such beliefs, often contradictory) judgments in reference to limited aspects of putative divine actuality, while only an omniscient being him/her/itself could know to what degree such human judgments approach omniscience. Thus the one maximally comprehensive truth accessible to any given sectarian group of living religious believers, at the level of their unavoidably finite understanding, is that they, in company with other groups of believers in various conflicting religious doctrines, have made the judgment that there is one or another omniscient god (limited aspects of which they understand). Even the judgment made by some believers that humans can in their post-death experience enter into a communion with an omniscient god whereby they too will attain complete understanding, remains only an anticipatory judgment made by mortals of finite judging capacity, and thus fails to exceed the bounds of the one maximally comprehensive finite truth available to living humans.

As presented thus far, the concept of one maximally comprehensive truth has had reference to what may be called informative judgments dealing with matters of fact or faith (whether in reference to the realms of the natural or the reputed supernatural levels of existence) as distinct from evaluative judgments dealing with the moral and aesthetic opinions of humans. But with little modification the concept applies as well to the latter types of judgment as to the former. There have been many instances of agreement among humans regarding questions of good and evil, and of beauty and ugliness, but also many cases of dissension; at no time have all humans coincided in their evaluative judgments, with those judgments being made in line with universally persuasive moral and aesthetic criteria (Hammer, 1966; Richter, 1967). There remains, however, the comprehensive informative judgment that all particular moral and aesthetic judgments have been made. One might offer as a maximally comprehensive evaluative judgment the assertion that it is good that all specific evaluative judgments have been exercised (that is, it is good that human beings have been creatures disposed to rendering differential moral and aesthetic judgments), but any meaning which even this evaluative metajudgment might have depends upon its prior assumption of the truth (informative metajudgment) that all evaluative judgments have indeed been made.

Although within the past-as-understanding many of the judgments (informative and evaluative) of given persons have been incompatible with those of other persons, human beings have nonetheless gained their measure of personal equilibrium, and societies have gained their measure of stability, through the circumstance that various judgments have with confidence been subscribed to in common by certain persons during given periods of time. In daily life the factor of mutual reinforcement of judgments by at least some segments of one's fellow human beings, on at least many items of common concern, rescues the human condition from one approximating universal insanity, in which no judgments would be sustained with any more assurance than any others. And certainly the individual who finds the concept of one maximally comprehensive truth to be persuasive will meanwhile have derived his or her own measure of personal equilibrium through accepting in common with at least some other persons the reliability of

certain judgments. There is no incongruity in the acceptance by an individual of various of the component judgments of the past-as-understanding together with the affirmation of the larger perspective of the one maximally comprehensive truth. However, the latter affirmation might be expected to increase for the individual in question the ratio of his or her own differential judgments that are maintained as hypothetical rather than true or false.

Perhaps we can anticipate some of the reservations which readers may have about the concept of one maximally comprehensive truth. Even those persons who may grant its cogency at the level of abstract discourse may perceive that truth, because it consists only of a metajudgment about the occurrence of other judgments, to be at best an empty truth. Such persons may remark that human living requires practical decisions and commitments in arriving at which humans must make specific judgments, but in the making of which judgments a grasp of the one maximally comprehensive truth could be of no help. These persons may further point out that the search for one maximally comprehensive truth seems to imply the desirability or need for one unified overarching truth, whereas the factor of continuing contradictory judgment-making may be the catalyst of creative progress in human understanding, and deserves thus to be encouraged rather than in some sense to be superceded.

We acknowledge the point of such reservations. In daily living we ourselves continually experience, of course, problems which cannot be solved, and satisfactions which can be neither clarified nor enhanced through reference to the one maximally comprehensive truth. Moreover, we place the same premium on creative contradiction as do other people. But meanwhile we find one type of satisfaction (along, surely, with some other persons of similar temperament) in producing a specific formulation of the inclusive reach of truth accessible to mortals. I submit that perception of the one maximally comprehensive truth marks the determinate limit of the indeterminate degree of human understanding; and any concept which identifies that limit cannot be philosophically trivial. Higher orders of living creatures, and most strikingly human beings, are inherently disposed to render judgments of true, false, and hypothetical; and indeed, the human attainment of prosperity, if not indeed the very survival of the human species, depends upon the skill with which they make those judgments. Meanwhile, perception of the one maximally comprehensive truth discloses, from the vantage point of greatest possible detachment, something fundamental about the total enterprise of human differential judgment-making; to apprehend that truth is to perceive the theoretical limit of human finite understanding.

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Biodiversity of Mothronwala Swamp, Doon Valley, Uttaranchal

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Abstract: India is a hub of biodiversity, encompassing a wide spectrum of habitats from tropical rain forests to alpine vegetation and from temperate forests to coastal wetlands. Among the 25 hotspots, India is considered as eighth hottest of hotspots extending from Western ghats on one side and Eastern Himalayas on the other. India contributes significantly to this latitudinal biodiversity trend with mere 2.4% of the world's area. Wetlands are transitional zones between the terrestrial and aquatic environment. These habitats perform major ecological role in the biosphere. Many of the fossil fuels are known to be produced and preserved by the swampy environment of the carboniferous period. These are source, sinks and transformers of a multitude of chemicals, biological and genetic materials. These produce a rich collection of plants, many of which are potential for one, or more economic use these provide food, timbers, fuel, fodder and forage etc. India has a rich variety of wetlands habitats. Tropical swamp forests once formed an important part of vegetation and extended all along the base of Himalayas from Assam to Peshawar. The International Biological Program (IBP) states that: "A wetland is an area dominated by specific herbaceous macrophytes, the production of which takes place predominantly in the aerial environment above the water level while the plants are supplied with amounts of water that would be excessive for most other higher plants bearing aerial shoots". Doon valley is known for its swamps. There was a time when low lying areas of the valley were having a chain of swamps but human interference once started in the name of "Malarias Climate" still persists. The trees were cut at that time and the openings created resulted in the extinction of most of the swamps. Wetlands are one of the most productive ecosystems and thus subjected to human greed which is yet another reason for their extinction. The Mothronwala swamp is a "Hot Spot" of biodiversity due to its topographic and edaphic variations. Unfortunately these habitats have not been explored from ecological point of view. The fresh water swamp of Mothronwala is under threat due to human interference and other anthropogenic activities. The present work was carried out to explore the biodiversity of the swamp and suggest conservation and management strategies. [Life Science Journal. 2006;3(2):73-78] (ISSN: 1097-8135).

Keywords: wetlands; swamps; biodiversity; Mothronwala; conservation

1 Introduction

Diversity is a concept about range of variation or differences among entities. The term biodiversity is a contracted form of biological diversity. Biodiversity is the degree of variety in nature and nature itself and also is the variability among living organisms from all sources including terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are a part. It includes diversity within species, between species and ecosystems. It is the most significant national asset and constitutes an enduring source for supporting the continued existence of human societies.

Wetlands are neither aquatic nor terrestrial, but are transitional zones. Swamps lie in the palustrine system of wetland. Swamps are marshy areas with typical habitats where water oozes out in perennial streams at constant level through out the year. They support characteristic vegetation on account of specialized edaphic conditions, as influenced by free water accumulation. Unfortunately these habitats have not been explored sufficiently from ecological point of view.

The Mothronwala swamp is a "Hot Spot" of biodiversity due to topographic and edaphic variations. The only authentic record of the area available is in the old settlement documents preserved in the office of the District Collector, Dehradun. The earliest document available is one - dated 1862, and on a map the site is indicated as "Land under water" and lies close to the Bindal River. A later record dates 1902 reveals that the river has changed its course and there is a wide gap between the present course of the river and the forest. Local enquires made of the village elders have elicited the information that in the past, the swamp was much deeper and more inaccessible than at present. The villagers dreaded approaching the swampy zone. In a report on the Dehradun forests prepared by Dr. G. King and published in 1871, a reference was made to these areas and it was recommended that

the forest department should drain the swampy places, which would incidentally improve the health of the eastern part of Doon but nothing appears to have been done in this regard.

The swampy zones are located in between the ridges and are composed of innumerable pools with characteristics bubbling and small intercommunicating streams. The northern portion, however, is drier than the southern, which is slushier and consists of loose soil. Besides the pools and streamlets mentioned above there are two large streams with a swampy base, which originate from the extreme north of the forest and flow from the north to south. In Doon valley there are many patches of freshwater swamps, which are recognized as integral part of wetland ecosystems. Kanjilal (1901) first emphasized on vegetation and botanical value of swamps. Vegetation and soil texture of Mothronwala have been studied by Dakshini (1960a, 1960b, 1965, 1970, 1974). Deva and Aswal (1974) studied the taxonomy and ecology of Mothronwala swamp. Deva (1974), Srivastava (1978) and Ghildiyal (1989) studied the vegetation of other swamps of Doon valley that include Golatappar and Manu swamp. However, study relating to the biodiversity of Mothronwala swamp has been left untouched, so the present study aimed to explore the biodiversity and give its conservation and management strategies.

2 Materials and Methods

2.1 Study area

Mothoronwala, Dulhani-1 (new reserve) and Navada 10 – 14 (old reserve) of Lachiwala range about 5 km from main city of Dehradun, at an elevation of 600 m above sea level. It occupies an area of approximately 22 acres. The swamp lies at 30° 15′ 40″ and 30° 16′ 45″ N latitude and 78° 1′ and 78° 2′ 15″ E longitude and lies to the South-East of Dehradun near the military township of Clement Town. On the East is the village of Mothronwala from which the swamp derives its name. On the north lies Banjarwala Tea Estate. On the West lies the Sushwa river, stream coming out of the swampy zone drains into the river that ultimately discharges into the Ganga through Rispana River. On the South is the Clement Town water works.

The swampy area of Mothronwala is humid and fairly green. The maximum rainfall ranges between 600 – 800 mm during the months of July – August and minimum is recorded during April – May. The maximum temperature reaches up to 40 ℃ during the months of May and June whereas minimum of 2 – 30 ℃ during December – January.

The ridge of Mothronwala swamp is about 10 – 11 m above the surrounding level. The slope along the ridge is approximately 20° – 30° . The northern part of the ridge is drier than the southern area, which is slushy. Inside the swampy area, the subsoil water level is quite high and remains so through out the year. The slush in marshy place is knee deep. During rains the water infiltrate through the gravelly soil extending over a very large area of the terrain oozes out here in a series of deep but narrow ravines giving rise to a number of streams which unites into a few main channels pour into the Suswa river.

2.2 Collection of aquatic flora and fauna

Clusters of algal filaments were collected from the swamp for the study of diatoms and algae present in them. Insects attached to stones were collected by a fine forceps. Insects inhabiting the shallow areas of the streams below stones were collected by enclosing 1 m^2 of the substratum with fine square-mesh netting cloth and sweeping the area completely. The insects were collected in cloth and picked up. The collected material was preserved in 4% formalin and identified.

2.3 Collection of terrestrial flora and macrophytes

Parts of different types of vegetation having flower, bud, node etc. were collected and then pressed in newspapers and dried for identification. The herbaria were identified at Botanical Survey of India (BSI), Dehradun.

3 Results

3.1 Plant diversity

Mothronwala swamp possesses peculiar vegetation due to topographic and edaphic varaiations. It has diverse and dense vegetation ranging from climbers and small herbs to tall trees. Indiscriminate human interference has led to the degradation of the swamp forest to a great extent leading a very small green cover. The original forest vegetation had dwindled to a larger extent and only two tree species namely *Shorea robusta* and *Dalbergia sisso* are left in the region. Other tree species like *Bischofia javanica*, *Celtris australis*, *Litsaea monopetala*, *Quercus leutrichophora*, *Toddalia asiatica etc.*, could also be seen on the few places. Exorbitant growth of *Lantana camara* and other exotic weeds have replaced the larger part of the vegetation. The shallow streambeds often extending over vast area of the swamp are covered with original hydrophytic and amphibious communities *Calamus tenuis* is the most dominant species. Shrubs in the swamp reach to a maximum height of

2 – 3 m. A pure community of *Ipmoea fistulosa* dominates upper portion of the swamp and bank of channel. The villagers collect *Rorripa nasturtium aquaticum*, observed as patches along the stream for vegetable. The herbaceous vegetation of the ridge is very sparse. The dominating ground vegetation is *Parthenium hysterophorus* and the grass *Cynodon dactylon*. On the ridges small tree communities like *Ficus palnata* and *Pyrus paschia* were common. *Mallotus philippensis*, *Indigofera tinctoria*, were found at few places. Invasive weed *Lantana camara* occupies most of the area. The dominantly vegetation was *Parthernium hysterphorus* and few grasses like *Cynodon dactylon*. Small trees like *Desmodium*, *Indigofera tinctoria*, *Ficus palnata* could be seen on the slopes. The surface of the slope is almost covered with large number of herbs like *Ageratum conyzoides* (Table 1).

Table 1. Plant diversity of Mothronwala swamp

Species	Name
Trees	*Shorea robusta*
	Dalbergia sissoo
	Celtris australis
	Ficus palmate
	Sapium sebiferum
	Solanum torvum
	Indigofera tinctoria
	Ficus religiosa
	Caryopteris wallichiana
	Pyrus pashia
Shrubs	*Ardisia solanacea*
	Mallotus philippensis
	Carrisa opaca
	Zizyphus mauritiana
	Murraya koenigii
	Smilax glaucophylla
	Plectranthes japonicus
	Rubus niveus
	Polygonum chinense
Weeds	*Lantana camara*
	Parthenium hysterophorus
	Eupatorium adenophorum
Herbs	*Argemone mexicana*
	Solanum nigrum
	Chenopodium album
	Rungia pectinata
Grasses	*Ageratum conyzoides*
	Cynodon dactylon
	Cyperus kyllingia
	Eleusine indica

In the swampy zone, the plant diversity varies according to the habitat, in pools and numerous streams usually macrophytes are found. Among shrubs *Ipomea fistuosa*, *Lantana camara etc.*, are commonly found. *Polygonium barbatum*, *Oenanthe javanica*, *Desmodium trifolium* are seen along the streams and present on well-drained soils.

The ground flora covers species like *Acorus calamus*, *Parthenium hysterophorus etc*, the livestock grazes the palatable species during the summer season, while the fern *Diplazium esculentum* locally known as lingora is collected for the vegetable in the region. *Calamus tenuis* is the most dominant at shallow streambeds and *Ipomoea fistuosa* is dominant in the upper portion of the swamp (Table 2).

Table 2. List of aquatic macrophytes of the swamp

Taxonomical name	Family
Ranunculus sceleratus	Ranunculaceae
Rorripa nasturtium aquaticum	Brassicaceae
Sida acuta	Malvacea
Sida cordata	Malvacea
Ventilago denticulate	Rhamnaceae
Acer oblongum	Acoraceae
Acer pennata	Acoraceae
Pyrus pashia	Rosacea
Carallia integerrima	Rhizophoraceae
Oenanthe javanica	Apiaceae
Oldenlandia corymbosa	Rubiaceae
Inula cappa	Arteracea
Enhydra fluctuans	Arteracea
Ipomoea carnea	Convolvulaceae
Ipomoea fistulosa	Convolvulaceae
Bacopa monnieri	Scropulariaceae
Lantana camara	Verbenaceae
Allmania nodiflora	Amranthaceae
Polygonum barbatum	Polygonaceae
Commelina berghalensis	Commelinaceae
Narengaporphyrowm	Poaceae
Imperata cylindrica	Poaceae
Coix lachrymal jobi	Poaceae
Acorus calamus	Araceae
Calanus tenuis	Arecaceae
Pouzolzia pertendra	Urticaceae
Canna indica	Cannaceae
Cyperus iria	Cyperaceae
Cyperus globosus	Cyperaceae
Scirpus eractus	Cyperaceae
Justicia quinqueargularis	Acanthaceae

A total of 19 genera of algae belonging to three orders were found in the stagnant water of the swamp. 16 species belonging to Bacillariophyceae, 2 species of Chlorophyceae and 1 species of Myxophyceae were found. *Tabelleria* of Bacillariohyceae was found to be abundant. Amongst the Chlorophyceae *Spriogyra* was found to be abundant (Table 3).

Table 3. Abundance of Algal components

Name	Abundance
Bacillaripophyceae	
Cymbella	+ +
Synedra	+ +
Pinnularia	+ +
Meridion	+ +
Diatoma	+
Achnathes	+
Gomphonema	+ +
Cocconeis	+ +
Melosira	+
Pinnularia	+
Nitzchia	+
Tabelleria	+ + +
Stauroneis	+
Flagilaria	+
Naviculla	+ +
Licmophora	+
Chlorophyceae	
Spirogyra	+ + +
Chlorella	+
Myxophyceae	
Oscillatoria	+ +

+ + + Abundant, + + Common, + Rare

3.2 Animal diversity

Biodiversity is key factor for natural development of global ecosystem. The concern for biodiversity has emerged as a result of quantification of consumers and consumables. Among the animals *Lepus nigricollis* (Indian Hare) and *Susscrofa cristatus* (wild boar) were known to be dominant. *Rana tigrina* the only amphibian was found abundant. Four species of fishes also represented the animal diversity (Table 4). Leeches are found in large number during the rains. Water snakes were common in the streams. Among the macro-zoobenthos 13 species belonging to 5 orders were identified. Amongst the 13 species of macroinvertebrate present 5 species represented genera Trichoptera, 2 species of Ephemeroptera, 2 species of Odonata, 2 species of Coleoptera and 2 species of Hemiptera. Three species of Molluscs also represented the animal diversity of the swamp. Amongst the Trichopterans, *Planaria* was found to be abundant whereas *Hydropysche* was found to be rare. *Ephemerall* of Ephemeroptera and *Gerris* of the order Hemiptera were also found abundantly (Table 5).

Table 4. List of fishes found in the stream flowing in the swamp

Vernacular name	Scientific name
Kali Machi	*Barbus chilinoides*
Baan	*Mastacembalus*
Sewal	*Vphicephalus punctatus*
Potto	*Barbus ticto*

Table 5. Abundance of Macroinvertebrates

Name	Abundance
Trichoptera	
Molanna	+ +
Hydropsyche	+ + +
Plannaria	+
Economus	+
Hydroptila	+
Coleoptera	
Amphizoa lecontes	+ +
Anchycetus	+
Molluscs	
Gyraulus	+ + +
Cerithidea	+ + +
Lymnaea	+ + +
Hempitera	
Gerris	+ +
Hespercorixa	+ + +
Ephemeroptera	
Heptagenia	+ +
Ephemerella	+ + +
Odonata	
Enallagma	+ +
Agrion	+

+ + + Abundant, + + Common, + Rare

4 Discussion

The threats to wetlands may be divided into two broad categories: natural threats and anthropogenic threats, which may be direct or indirect. Natural threats include eutrophication, erosion, storm damage, drought or biotic interference other than by man, which may lead to destruction of wetlands. The human intervention by drainage and reclamation for agriculture and urban construction

stop them to play their usual ecological roles. Ecological degradation of wetlands together with pollution has resulted in the loss of flora and fauna.

The fresh water swamp of Mothronwala is under great environmental stress and has been degraded to a great extent during the last few decades. The major portion of the swamp has been encroached upon by the human settlements, agriculture, cultivation and related developmental activities. Forests felling are common on the ridges. The villagers have occupied the peripheral area of cultivation of various fodder species. As the cantonment is in the close vicinity of the swamp, the area is being exploited to meet out the various needs of the military persons. A water pump has been installed inside the swamp to pull out the water to be used for drinking, bathing and other domestic purposes.

Lopping of trees by people from neighboring village results in the deformity of some of the trees with the consequent effect on the ground floor vegetation. Invasion of exotic weeds like *Lantana camara*, *Parthenium hysterophorum*, *Ageratum conyzoides*, *Ipomoea* has drastically changed the vegetation of the swamp. Plant species like *Shorea robusta*, *Bombax ceiba*, *Grewia oppositifolia*, *Toona ciliata* are used for fodder, fuel and timber by villagers.

Cattle trampling is another big biotic factor responsible for reduced vegetal cover of the region. Grazing is also a factor to be considered particularly on the slope and the ridges. Leasing out of medicinal plants like *Centella asiatica*, *Bacopa monnierii*, *Berchemia floribunda*, *Desmodium triangulare*, *Cassia pumila*, *Acorus calamus etc.*, have caused the depletion of these species from the swamp area. At Mothronwala swamp, the ecological succession is resulting into conversion of aquatic region to terrestrial is also contributing to the shrinkage of waterbed area. Erosion of the exposed slopes is responsible for the alteration in vegetational cover from season to season. Higher deforestation rate results in the loss of topsoil, which is drained off with rainwater and settles down in the stream. This result in rise of soil level in swamps making them much shallower with reduced water spread area.

Wetlands are the sources sinks and transformers of chemical, biological and genetic materials. They play a significant role in environment by providing a unique habitat for a wide variety of flora and fauna. However, over a period of time these natural heritages are continuously disturbed by human interference and over exploitation of biological resources available in them or in nearby locations. Since last few decades efforts have been made at national and international level to assess the status, management and conservation of wetlands with growing awareness the importance of these fragile ecosystems have been realized throughout the globe (Chatrath, 1992).

The long-term solution to the problem of protecting wetlands lies in educating the masses. Unless people realize the need to safeguards wetland ecosystem and are made aware of how they can contribute to this effort, there is little hope for the survival of these ecologically valuable and vulnerable habitats. The fresh water swamp of Mothronwala is under threat due to human interference and other anthropogenic activities. As a consequence, some measures are of utmost importance to check their further deterioration like the knowledge of the physical dimensions of these fresh water swamps by way of field surveys and other appropriate techniques like remote sensing etc. should be gained.

Inventory of both flora and fauna in these swamps should be made and rare, endangered and economically important species should be given top priority for their protection. Since deforestation in the catchment area due to human interference, has adversely affected these swamps. It is necessary to go for large scale afforestation in these areas.

Sincere efforts should be made to check the soil erosion from slopes, which lead to siltation in these swamps. It can be done by constructing check dams in high reaches, at different places and initiating afforestation in these areas. There should be a regular testing and monitoring of the water quality of these swamps. The water samples need to be taken from the disturbed areas along the stream at regular intervals to judge the adverse effects of human activities. State Pollution Control Board situated locally should be entrusted with such responsibility.

There should be a complete ban on all construction activities up to a specified distance, say about 100 m or more from the swamp. This can be ensured by making a clearance mandatory from the state environment department before undertaking any construction activity in the vicinity of the swamp. Efforts should be initiated by the State Forest Department to protect these swamp forests from further destruction by enforcing strict laws and warding heavy penalties on defaulters who are harming these ecologically sensitive zones by over exploitation of resources, cutting and lopping, diversion of water for irrigation and agriculture and urban land use.

To make people aware of the importance and threats to wetlands and their conservation, various government institutions, NGOs and media (both

print and audiovisual) should take the lead and make it a mass movement. Local communities should be involved to ensure sustainability of conservation effort under taken by the government agencies. For this they can be involved in decision-making processes required for management and conservation of wetlands.

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QTLs for Flag Leaf Area of Rice under Multi Environments

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Abstract: QTLs with epistatic effects and environmental interaction effects for flag leaf area of rice were studied by mixed-model based QTL mapping with a doubled haploid population from IR64/Azucena in four years. The results revealed that many QTLs were involved in epistasis, and the same locus could get involved in interactions with more than one other locus. Such loci might play the role of modifying agents that tend to activate other loci or modify the action of other loci. QTL by environment (*QE*) interaction effects were detected more often than QTL main effects for flag leaf area, as might indicate that gene expression could be greatly affected by environmental factors for this quantitative trait. [Life Science Journal. 2006;3(2):79-82] (ISSN: 1097-8135).

Keywords: quantitative trait locus; epistatic effects; QTL by environment interaction effects; flag leaf area of rice

Abbreviations: DH: double haploid; QE: QTL × environment; QTL: quantative trait locus; LA: leaf area

1 Introduction

Flag leaf area is very important for grain production in rice and is genetically controlled by quantitative genes. So genetic analysis and quantitative trait locus (QTL) mapping has been conducted; some QTLs and their effects were revealed in one environment[1-3]. But in heritance of quantitative traits, gene expression could be modified by epistatic interaction with other genes and by environmental factors[4]. To dissect the quantitative inheritance of flag leaf area in rice, the QTL mapping method based on mixed linear model approaches and the software QTLMapper[5,6] were employed for detecting QTLs with additive and epistatic effects as well as their QTL by environment (*QE*) interaction effects.

2 Materials and Methods

A population of 123 double haploid (DH) lines derived from a cross between an irrigated *indica* variety IR64 and an upland *japonica* variety Azucena[7] were used in the experiments. The genetic map of this population containing 175 markers distributed among 12 chromosomes covering 2005 cm with an average distance of 11.5 cm between markers[8] was used for QTL mapping.

The 123 DH lines and their parents, IR64 and Azucena, were grown in a randomized complete design with two replications at both Hainan of China in 1995 and Hangzhou of China in 1996, 1997 and 1998. Hainan Island is located in the Southern China Sea at 18° north latitude while Hangzhou is located in Eastern China at about 30° north latitude. These two places show great difference in climate, soil conditions, day length, and even rice growing seasons. At Hangzhou, there were remarkable divergences of temperature, soil conditions among the three years. The experiment was conducted from early December 1995 to late April 1996 at Hainan where rice can grow well all year round. At Hangzhou, experiments were carried out from late May to early November in 1996, 1997 and middle May to middle October in 1998. In all environments, the germinated seeds were sown in a seedling bed and the seedlings were transplanted to a paddy field 30 days later, with a single plant per hill spaced at 15 cm × 20 cm. Each plot included four lines with eight plants per line. At the maturity stage, length and width of flag leaf of 6 central plants in each plot were measured. The flag leaf area (LA) was calculated according to Yoshida[9] et al: Leaf area = Leaf length × Leaf width × 0.725.

QTLs as well as their environmental interaction effects were mapped by the mixed model based QTL mapping approach and software of QTLMapper[5,6]. The likelihood ratio value of 11.5, which is equal to a LOD score of 2.5[10], was used as a threshold to declare the detection of QTL or epistasis.

3 Results and Analysis

3.1 Transgressive segregation of leaf area

The phenotypic behavior of leaf area for the DH population and its parents under four environments were described in Table 1. Leaf area (LA) of parent Azucena was larger than that of IR64 in all environments. Wide variation from maximum to minimum values occurred among DH lines across all four environments. But also the population segregated continuously like normal distribution, both skew and kurt values were less than 1.0, as suggested that the DH population were suitable for QTL analysis.

Table 1. Phenotypic behavior of flag leaf area under four environments

Environment	Parents		DH population					
	IR64	Azucena	Mean	Max	Min	Stdev	Skew	Kurt
Hainan in 95	14.9	30.1	27.97	51.42	11.66	8.05	0.34	−0.33
Hangzhou in 96	29.7	52.5	42.49	80.96	17.12	13.4	0.47	−0.43
Hangzhou in 97	32.9	57.1	45.13	70.86	20.67	10.2	−0.01	−0.82
Hangzhou in 98	31.0	37.3	32.26	55.00	21.33	5.88	0.72	0.59

Note: Mean, Max, Min, Stdev, Skew and Kurt are the average, maximum, minimum, standard deviation, skew and kurt of all observations for DH lines in one environment.

3.2 Quantitative trait loci for LA

Altogether 15 QTLs for leaf area with additive effects and/or additive × additive epistasis effects were found on 10 chromosomes of all the 12 chromosomes (Table 2). They were named for leaf area as "La" with the chromosomal number.

Table 2. Positions of QTLs with additive effect and/or additive × additive epistasis effect for flag leaf area

Chrom.	QTL	Marker interval	Distance(M)
1	La1 − 1	RG246 − K5	0.1
1	La1 − 2	RZ730 − RZ801	0.08
2	***La2 − 1***	***RG157 − RZ318***	***0.14***
2	La2 − 2	RZ123 − RG520	0.1
3	La3 − 1	RG348 − RZ329	0
3	*La*3 − 2	*RZ*403 − *RG*179	0.04
3	La3 − 3	CDO87 − RG910	0.02
4	La4	RZ590 − RG214	0
5	La5	RZ70 − RZ225	0.18
6	La6 − 1	RZ667 − Pgi-2	0
6	La6 − 2	Amy2A − RG433	0.02
7	La7	PGMS007 − CDO59	0.04
9	La9	RZ228 − RZ12	0
10	La10	RG134 − RZ500	0
12	La12	CDO344 − RG958	0

Note: QTLs with both detectable additive effects and epistatic effects were presented in regular form while the QTLs involved in epistasis but without detectable additive effects were presented in bold italic form, and the QTLs with only additive effects but no epistatic effects were notified with underling lines.

Table 3. Additive and/or additive × environment interaction effects of QTLs across four environments

QTL	*a*	*ae1*	*ae2*	*ae3*	*ae4*
La1 − 1	−1.75**	1.94**		−1.78**	
La1 − 2		−1.17**	−0.95**	3.87**	−1.75**
La2 − 2		1.44**	0.68**	−2.76**	0.64**
La3 − 1		1.61**	−2.79**	2.77**	−1.60**
La3 − 3	−1.63**				
La4	−4.91**		−2.55**	−1.29*	2.78**
La5	−2.01**		−2.76**		1.95**
La6 − 1		0.72**	−4.58**	1.35**	2.52**
La6 − 2	1.38**		3.72*		
La7	1.90**	−3.44**	3.32**		
La9					0.51**
La10		−0.35*			
La12			−0.82**		

Note: *a*, *ae1*, *ae2*, *ae3*, *ae4* represent additive main effect and additive × environment interaction effect at Hainan in 1995, at Hangzhou in 1996, 1997 and 1998, respectively. * and ** represent the significance level of $P = 0.01$ and $P = 0.005$, respectively.

If there were more than one QTL in a chromosome, the serial number was added after chromosomal number separated by a hyphen. The positions of these QTLs were indicated by the marker interval bracketing the concerned QTL with the estimated distance in morgon (M) from the left marker. The 11 QTLs with both detectable additive effects and epistatic interaction effects were presented in regular form, while the 2 QTLs involved in epistatic interactions but without detectable additive effects were presented in bold italic letters, and the other 2 QTLs with only additive

effects but no epistatic effects were notified with underling lines. The estimated additive effects and the additive × additive epistatic effects at significance level of 0.01 or 0.005 under different environments were presented in the Table 3 and Table 4, respectively.

Table 4. Epistasis and epistasis by environment interaction effects of QTLs across four environments.

QTLi	QTLj	*aa*	*aae1*	*aae2*	*aae3*	*aae4*
La1-1	La1-2		0.47**			
La1-1	La6-1			0.62**		
La1-1	La10	1.15*				
La1-2	***La3-2***	1.03**				
La1-2	La5		0.37*			
La1-2	La7		-0.51*			
La2-1	La2-2	1.10*				
La2-1	La12		-2.33**	3.57**	-5.16**	3.93**
La2-2	La6-1	-1.38*				
La2-2	La7		0.54**	-2.01**	-1.34**	2.81**
La3-3	La4		1.21**	-1.50**		
La3-3	La6-1	-2.11**	1.28*	-3.48**		1.94**
La3-3	La9	-1.41**	5.12**	-3.19**	-1.92**	
La9	La10	1.39**				

Note: *aa*, *aae1*, *aae2*, *aae3*, *aae4* represent epistatic main effect and epistasis × environment interaction effect at Hainan in 1995, at Hangzhou in 1996, 1997 and 1998, respectively. * and ** represent the significance level of $P=0.01$ and $P=0.005$, respectively.

3.3 Analysis for QTL additive effects

13 QTLs with additive main effect (*a*) and / or additive by environment interaction effect (*ae*) were shown in Table 3. In them, 5 QTLs had both *a* and *ae* effects, while 7 QTLs with only *ae* effects in one to four environments and 1 QTL with only *a* effect. As to QTLs' *a* effects, 4 QTLs had contribution to decreasing leaf area and 2 QTLs to increasing. As to QTLs' *ae* effects, usually QTLs had opposite directions of *ae* effects in two or more environments. The additive main effect *a* was the accumulated effect expressed in the same way across different environments, while the interaction effect *ae* was the deviation due to specific environment. At a specific environment, the total effect of a QTL should include the main effects plus *QE* interaction effects at that environment. The *a* effect of QTL La4 reached maximum absolute value 4.91 cm^2, and *ae* effect from QTL La6 - 1 reached maximum absolute value 4.58 cm^2 in 1996. Maybe the environment in 1996 could influence the QTL La6 - 1 greatly. The results of *ae* effects were obviously more often detected than *a* effects, which might also suggested for quantitative traits gene expression could be modified by environmental factors easily.

3.4 Analysis for QTL epistatic effects

Altogether 14 digenic epistatic pairs with epistatic main effect (*aa*) and/or epistasis by environment interaction effect (*aae*) were detected (Table 4). Among them, only 2 pairs had both *aa* and *aae* effects, while 5 pairs had only *aa* effects and 7 pairs had only *aae* effects. The maximum absolute magnitude of *aa* and *aae* effects reached 2.11 cm^2 to 5.16 cm^2, respectively. The range of epistasis × environment interaction effects was wider than the range of epistasis main effects, and epistasis × environment interaction effects were more often detected than epistasis main effects, which might indicate that digenic interactions were more easily subjected to environmental influence.

The composition of epistatic pairs was interesting for that, the detected pairs included 2 QTLs without detectable *a* or *ae* effects (notified in bold italic form in Table 4). The role of this kind of QTL might be only to regulate other QTL. Another noteworthy case was that it was fairly common for one QTL to interact with more than one QTL. This also indicated the possibility of multi-QTL associations in the formation of complex traits.

4 Discussion

Both epistatic effects and QTL × environment interaction effects are important components of genetic basis for complex traits. But many of researches have been based on models assuming no epistatic effects or *QE* interaction effects[1-3]. This usually cannot give unbiased estimation for QTL parameters. Partitioning of epistasis from other genetic components of variation would help to obtain more reliable results of QTL mapping. Furthermore, the contribution of QTLs to the trait should also vary according to the growing environment. Usually, *QE* effects are treated as random effects especially in different years. They imply the extents that QTLs would be affected by unknown environment factors in different years.

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Wheat Milling By-products Fermentation: Potential Substrate for Bioethanol Production

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Abstract: An overview on the potential application of wheat milling by-products for bioethanol production is made. The fermentation performance of low-grade wheat flour (LG) and wheat bran (WB) was evaluated and compared to wheat flour (WF). α-amylase or cellulase was used for liquefaction, followed by simultaneous saccharification and fermentation (SSF) by glucoamylase and *Zymomonas mobilis*. The final ethanol concentration, overall productivity and yield obtained from LG (51.4 g ethanol/L, 2.72 g ethanol/L·h and 0.17 g ethanol/g flour, respectively) were considerably higher compared to WB (18.1 g/L, 1.09 g/L·h and 0.02 g/g). High LG fermentation rates, reaching the highest ethanol productivity (4.4 g/L·h) within 6 h of SSF, indicated considerable savings on fermentation time, compared to current industrial processes. [Life Science Journal. 2006;3(2):83-87] (ISSN: 1097-8135).

Keywords: wheat; flour; by-product; bran; fermentation; ethanol

Abbreviations: LG: low-grade wheat flour; μ: growth rate; P: ethanol production; Q: ethanol productivity; Q_V: overall volumetric ethanol productivity; R_P: solid residue; SSF: simultaneous saccharification and fermentation; WB: wheat bran; WF: wheat flour; Y_L: liquefaction yield; $Y_{P/S}$: ethanol yield

1 Introduction

With the search for alternative renewable energy sources, biofuels are becoming a viable solution, as they are non-fossil fuels from renewable sources. Of all biofuels, ethanol is already produced on a fair scale worldwide. The bulk of the production is located in Brazil and the USA.

Various studies on ethanol conversion systems from wheat products have been conducted based on the utilization of raw wheat flour or damaged wheat grains (e.g. Montesinos & Navarro 2000, Suresh et al. 1999). However, only a few reports on wheat milling by-products fermentation are available (e.g. Adrados et al. 2005). Thus, the objectives of this study were to develop a simultaneous saccharification and fermentation (SSF) process in batch mode for wheat milling by-products and to evaluate the fermentation performance of low-grade wheat flour (LG) and wheat bran (WB), compared to wheat flour (WF).

2 Materials and Methods

2.1 Materials

Raw material: LG, WB and WF. Typical composition was summarized in Table 1.

Bacterial cells: *Zymomonas mobilis* NBRC 13758.

Enzymes: α-amylase (51 U/mg, Sigma, USA); glucoamylase (23 U/mg, Sigma, USA); cellulase (106 U/mg, MP Biochemicals).

Liquefaction: One liter slurries containing 200 g dried matter/l of LG (pH 6.1), WB (pH 5.9) or WF (pH 5.7) were hydrolyzed separately using 400 U α-amylase/g flour (in the case of LG or WF) or cellulase (for WB) at 55 ℃, 100 rpm for 2 h.

SSF: 200 U glucoamylase/g flour and 100 ml starter culture (3.1×10^8 viable cells/ml) were added to the liquefied slurry and the SSF was conducted in a previously sterilized 2 liter jar fermentor (Marubishi) at 35 ℃, 100 rpm, pH 4.5 in anaerobic environment sparging N_2 gas (100 ml/min).

2.2 Analytical methods

Glucose, maltose, and ethanol were deter-

mined using HPLC (Shiiba et al. 1993). The reducing sugars were calculated based on the sugar distribution obtained by HPLC. They were composed of monosaccharides (glucose, arabinose and xylose) or disaccharides (maltose and cellobiose). The liquefaction yield (Y_L) was calculated as the quotient of the amount of maltose released during liquefaction by the initial substrate.

The fermentation performance was evaluated based on ethanol concentration (P), productivity (Q), overall volumetric productivity (Q_V), yield ($Y_{P/S}$), and solid residue (R_P). Q_V was calculated as the quotient of the total ethanol production by the fermentation time. $Y_{P/S}$ was defined as the quotient of the ethanol produced by the initial substrate. An aliquot of the final product was centrifuged (4, 000 rpm, 20 min), and the solid residue dried in order to evaluate the residue formation (R_P).

Table 1. Fermentation performance of various wheat milling products

Substrate	LG	WB	WF
Raw material			
Substrate concentration (g/L)[a]	200	200	200
Protein (%) (w/w)[b]	15.0	13.3	10.4
Starch (%) (w/w)[c]	15.6±0.09	11.7±0.25	62.0±0.04
Moisture (%) (w/w)	14.0±0.15	12.2±0.19	13.1±0.01
Ash (%) (w/w)	2.7±0.01	5.6±0.04	0.6±0.15
Final product			
Ethanol concentration (P)(g/l)[d]	51.40±0.37	18.10±3.17	68.10±1.43
Supernatant (ml)	600	320	760
Solid residue (R_P) (g dry matter)	28.50±2.69	111.20±8.31	8.30±0.93
Ash (%) (w/w)	3.90±0.04	18.70±0.21	7.70±0.42
Ethanol yield ($Y_{P/S}$) (g ethanol/g flour)	0.17±0.01	0.02±0.08	0.30±0.03
Overall volumetric ethanol productivity (Q_V) (g/L·h)	2.72±0.04	1.09±0.21	3.64±0.08
Growth rate (μ)(1/h)[e]	0.119	0.142	0.043

[a] 1 liter slurries were prepared for each substrate, separately

[b] Protein content (total nitrogen×5.7); provided by Nisshin Flour Milling Co. Ltd.

[c] Composition assays performed as described elsewhere (Neves et al. 2006). Mean±S.D. of three experiments

[d] Before centrifugation (5,000 rpm for 30 min)

[e] Growth rate was calculated by linear regression of microbial growth curves (Slope=μ/2.303)

3 Results and Discussion

3.1 Liquefaction

The LG liquefaction yield was 0.065 ±0.002 g maltose/g flour (Mean ± S.D., n = 3 repetitions), followed by WB (0.010 ±0.005 g/g), compared to the reference substrate WF (0.126 ± 0.021 g/g).

The Y_L from LG was nearly six fold that from WB, evidencing the lower initial starch content in WB. Besides releasing glucose (the major product of WB hydrolysis using cellulase), arabinose and xylose may also be produced, as mentioned in previous literature (Adrados et al. 2004; Shiiba et al. 1993). The sugar content during WB liquefaction fluctuated considerably between replicates. This behavior was likely caused by WB pentosans. They are known to have low water solubility, thus decreasing their availability for enzymatic hydrolysis (Adrados et al. 2004). For instance, while amylopectin is soluble in water, the starch granules itself as well as amylose are insoluble in cold water.

In the case of LG, maltose concentration was nearly stable after 2 h liquefaction, indicating the end of starch hydrolysis. This is in line with the results reported by Montesinos et al. (2000). They demonstrated that liquefaction during 2 h was necessary for efficient hydrolysis of glucose polymers when slurries containing 300 g raw wheat flour/L were hydrolyzed at 95 ℃ using thermostable α-amylase.

3.2 Simultaneous saccharification and fermentation

The results of SSF using different wheat products are depicted in Figure 1. The increase in glucose at the SSF onset implies that glucoamylase ac-

tivity was sufficient to release more glucose than *Z. mobilis* could metabolize. However, in the case of LG, after 2 h SSF the glucose level decreased continuously, disappearing at *c. a.* 9 h. Meanwhile, ethanol increased gradually, stabilizing at nearly 18 h.

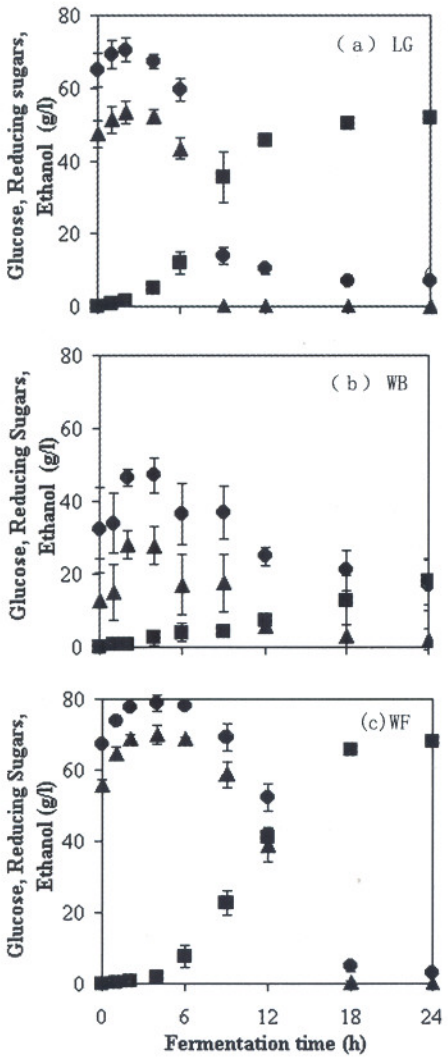

Figure 1. Time-course profiles of simultaneous saccharification and fermentation from various wheat products.
(a) Low grade flour; (b) Wheat bran; (c) Wheat flour.
Symbols: ▲, Glucose; ●, Reducing sugars; ■, Ethanol.
The bars represent the Standard Deviation ($n=3$ replicates)

From Figure 1c it was apparent that besides glucose, other reducing sugars were released during WB hydrolysis, mostly arabinose or xylose, rather than maltose which was rarely detected. These results are in agreement with Shiiba et al. (1993). Those authors reported that hemicellulose (which is mainly consisted of arabinose and xylose) is the major component of WB cell wall polysaccharides. Xylose fermentation involves a metabolic pathway with two enzymes, xylose reductase and xylitol dehydrogenase. *Z. mobilis* is not able to produce these two enzymes, thus the impossibility to ferment pentoses. Some other microorganisms, such as *P. stipitis*, produce these enzymes naturally (Kodaki et al. 2004). Slight amounts of cellobiose, a reducing sugar obtained by partial cellulose hydrolysis, were detected in WB fermentation mash; this is a potential carbon source, since it can be converted to glucose by α-glucosidase, which occurs naturally in wheat products.

Experimental microbial growth rate (μ) values indicated that *Z. mobilis* grew faster in WB- or LG-based substrates, rather than in WF. Low μ values obtained for WF (Table 1) suggested that in this case the sugars contained in the fermentation mash were preferably metabolized into ethanol, rather than used for biomass production, substantiated by high P and low R_P values.

3.3 Fermentation performance

The fermentation performance was evaluated using various parameters (P, Q, Q_V, $Y_{P/S}$, and R_P), summarized in Table 1.

Figure 2 shows the ethanol productivity (Q) profiles throughout the SSF process. Q values for WB were lower compared to LG or WF, which was likely due to the presence of pentose sugars in WB. Incomplete starch hydrolysis during WF liquefaction may have caused delay on ethanol production, as well. This was consistent with the hypothesis that starch hydrolysis is made difficult by the increased viscosity during liquefaction, caused by starch granules swelling as well as water penetration (Montesinos & Navarro 2000).

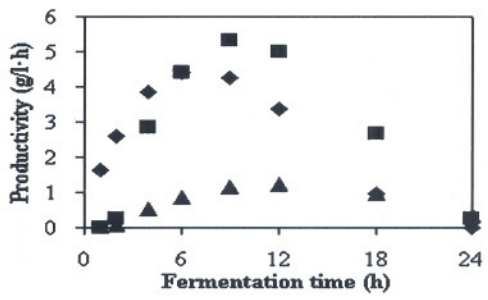

Figure 2. Ethanol productivity (Q) from various wheat products (◆, low grade flour; ▲, wheat bran; ■, wheat flour)
Q was calculated by differentiating the experimental ethanol production data as function of time (Jain et al. 1985).

To gain some perspective on the improvements achieved using different conversion systems and microorganisms, a comparison was provided in Table

2. A combination of high ethanol levels and productivity is desirable in minimizing processing costs. It was apparent that ethanol concentration and yield obtained for LG were relatively low, compared to other systems using WF. Such problems can be overcome, e.g. using fed-batch culture systems, as reported in previous literature (Roble et al. 2003). Those authors were able to reach up to 90 g ethanol/L using a circulating loop bioreactor for SSF of raw cassava starch.

In the year 2000 nearly 6.8 million tons of WF were produced in Brazil (FIBGE, 2001) resulting in about 0.34 million tons of LG. Assuming that this by-product could be fully used as feedstock, the potential bioethanol production from LG becomes 78.2 mega liters.

Table 2. Summary of ethanol production using various substrates and microorganisms

Reactor (volume)	Mode	Culture	Substrate (g/L)	Ethanol (g/L)	Productivity (g/L·h)	Yield (g/g flour)	Source
Jar fermentor (2 L)	Batch (40 h)	Commercial α-amylase, glucoamylase and *S. cerevisiae*	Raw wheat flour (300)	67	3.19	0.45[a]	b
Erlenmeyer (500 mL)	Batch (90 h)	α-amylase (from *B. subtilis*) and *S. cerevisiae*	Fine-wheat flour	44	0.49	0.18	c
			Damaged wheat	34	0.38	0.14	
			Damaged sorghum (250)	27	0.30	0.11	
Circulating loop reactor (9 L)	Fed-batch (600 h)	α-amylase (from *A. awamori*) and *S. cerevisiae*	Raw cassava starch (150)	90	1.17	0.45[a]	d
Jar fermentor (2 L)	Batch (48 h)	Commercial α-amylase, glucoamylase and *Z. mobilis*	LG (200)	51	2.72	0.17	This work
			WB (200)	18	1.09	0.02	
			WF (200)	68	3.64	0.30	

[a] Ethanol yield (g ethanol/g starch); [b](Montesinos et al. 2000); [c](Suresh et al. 1999); [d](Roble et al. 2003)

4 Conclusion

In this work, two wheat milling by-products, LG and WB, were utilized as substrate for bioethanol production. High LG fermentation rates, reaching the highest ethanol productivity (4.4 g/L·h) within 6 h of SSF, indicated considerable savings on fermentation time, compared to current industrial processes.

The present system employing commercial enzymes might be too expensive for commercial bioethanol production, in view of the current market prices of most amylolytic enzymes. This process was used to evaluate the fermentation performance of different wheat milling by-products. Industrial application of this system requires modifications in order to decrease the overall cost, e.g. *in situ* α-amylase production as well as immobilizing the ethanol-producing strain, utilizing fed-batch or continuous fermentation process.

In order to use wheat milling by-products for large scale fermentation, a system for collecting these by-products from the milling site has to be developed, which could be done after LG is recognized as a low cost feedstock.

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Medical Waste Management Practices in Thailand

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Abstract: Inappropriate medical waste management practices (WMPs) can cause hazards and risks that affect not only the generators and operators but also the general community. An investigation of WMPs in hospitals in Thailand has shown similar and different patterns of medical WMPs consisting of infectious waste, solid waste and hazardous waste, including wastewater practices. The quantity of infectious waste generated from different size of hospitals in Chiang Mai is at a rate of 0.17 kg/d/bed to 0.97 kg/d/bed. Most hospitals have an incinerator where medical waste is burned. The ash from the incinerator should be properly disposed in a landfill. Solid waste in hospitals is sent to a municipal landfill. In some hospital, there is a thriving recycling program. Some hazardous waste is either burned or sent to a private secured landfill. Others are collected in hospitals and stored for disposal. However, the assigned government agencies and a manual for helping them solve waste problems are needed. All hospitals have wastewater treatment plants (WWTP). Some WWTP need advice for coping effectively with the WWTP problems. In order to provide more effective WMPs in hospitals, a standard operating procedure (SOP) and regulations for segregation of infectious waste, solid and hazardous wastes must be developed. The SOP should outline the method for handling hospital wastes, how to collect, segregate, treat and dispose of these wastes. Furthermore, the agency responsible for regulating incinerators and WWTPs in the hospitals should regularly visit and inspect these facilities for improving their efficiency and solving problems when they occur. [Life Science Journal. 2006;3(2):88-93] (ISSN: 1097-8135).

Keywords: waste management practices; medical waste; regulations; standard operating procedure

Abbreviations: SOP: standard operating procedure; WMPs: waste management practices; WWTP: wastewater treatment plants

1 Introduction

According to the World Health Organization (WHO), the waste produced by health care facilities carries a higher potential for hazards and risks from infection and injury than any other kind of waste[1]. Medical waste or hospital waste consists of infectious, radioactive and toxic substances, as well as unsafe material from activities in clinics and laboratories in hospitals. It includes human blood and blood products wastes, tissues, animal wastes, microbiology laboratory wastes, radioactive and chemical wastes, pharmaceutical, sharp wastes in addition to general waste like paper, food and plastics[2]. Based upon the WHO 1994 report of hospital waste in America, Netherlands, and France, about 85% of the hospital wastes are actually non-hazardous waste, 10% are infectious waste and 5% are non-infectious waste but hazardous wastes. Although infectious and hazardous wastes from hospitals occur in small quantity of waste, there is a high potential of serious threat to spread out various diseases and hazardous materials from these wastes due to improper disposal of dumping and burning. The poor management of medical waste poses risks to public health and the environment, especially, in terms of the transmission of disease by viruses and microorganisms, contamination of underground water tables by untreated medical waste in landfills, as well as contamination of ambient air by uncontrolled burning. The problem of medical waste disposal in hospitals has become an issue of increasing concern, prompting hospital administration to seek new ways of safe and cost effective management of the waste, and keeping their personnel informed of the advances in this field. In Thailand, the estimated quantity of infectious waste by Pollution Control Department (PCD) in 2000 was reported to be a total of 13,250 tons or 36.1 ton/day[3]. These are generated from both government and private hospitals. Of 22 tons of infectious wastes are

generated in the regional part while about 24.1 tons of these wastes are generated in Bangkok and the nearby area.

1.1 Waste management program

The concept of waste management program has been introduced to many activities, namely, industries, business and commercial, communities, houses, and hospitals. It includes waste minimization, reduce/reuse and recycle, incinerator and landfill. The adoption and promotion of this program are still very limited in the hospitals because accrediting system is not functioning due to some barriers, such as government regulations on medical waste management, lack of support from hospital administrators, and poor environmental awareness. Therefore, the commitment of hospital directors to better management is the most important criterion in promoting the waste management program to hospitals. Without support from the hospital directors, good government regulations and better environmental awareness of the staff, introduction of waste management program will be difficult.

1.2 Waste management procedures

It consists of waste collection and segregation, storage, transportation, treatment and disposal[4]. Segregation of waste is the most important step in the process of waste management. It allows management of small quantities of waste thereby reducing the risks as well as cost of handling and disposal of a large mingled waste. Segregated waste need to be stored in identifiable containers, and must separate infectious waste from hazardous waste. The general waste or solid waste and non-hazardous waste transportation should be kept separate from infectious waste and hazardous waste. The transportation containers should be properly enclosed. The driver must be trained to follow established procedures in case of traffic accidental spillage[5].

1.3 Medical waste treatment technologies

There are several medical waste treatment technologies, namely, mechanical, thermal, chemical, and irradiation processes[4,6,7].

Mechanical process is used to change the physical form of the waste to facilitate waste handling. It consists of compaction and shredding. Compaction involves compressing the waste into containers to reduce its volume. Shredding is used to break the waste into smaller piece. This process is not considered acceptable for medical waste treatment by itself.

Thermal process is designed to use heat at low temperature (< 150 ℃) and high temperature (600 – 5,500 ℃) to decontaminate medical waste. The thermal processes include autoclaving, microwave treatment, and incineration. Autoclave is a steam sterilization technique that uses steam to contact with the waste directly to disinfect the waste. Microwave treatment is designed to use the electromagnetic radiation spectrum lying between the frequencies 300 and 300,000 MHz to inactivate microbial organism. Incineration processes use high temperature (800 – 1,050 ℃) combustion under controlled conditions to convert wastes containing infectious and pathogenic material to inert material residues and gases. It gives a significant volume and weight reduction and it sterilize the waste. There is limitation of the incinerator due to the occurring pollution during operation. It is needed to control its temperature.

Chemical process involves the use of chemicals like chlorine compounds for disinfection. This system needs shredding step in order to provide sufficient control between the waste and disinfectants.

Irradiation process is designed to use ultraviolet or ionizing radiation for irradiating and sterilizing the medical waste.

Among these technologies, autoclave and incinerator methods are mostly used as a sequence for treatment and disposal of infectious waste.

1.4 Laws and regulations for waste management

In Thailand, there are 4 major Acts related to hospital waste, namely, Public Health, Industry, Factory, and National Enhanced and Preservation of Environmental Quality Act, 1992[8].

2 Objectives

Through this study, the assessment of existing WMPs and the quantification of medical waste generated in Chiang Mai hospitals were performed in order to propose the best WMPs of hospitals.

3 Materials and Methods

The data were collected by surveying and interviewing the key informants who respond to waste management in hospitals and using a questionnaire which broadly included information on the type, quantity of waste generated and existing disposal, including all WMPs. The categorization of the hospital waste was attempted according to the Ministry of Public Health Notification for Infection Waste Managementm, 2002, and the related regulations under the 4 major Acts as mentioned above. The study was carried out in hospitals in Thailand in 2004 as shown in Table 1.

4 Results and Discussion

The waste management procedures and prac-

tices in hospital are shown in Table 2. The sources of hospital waste come from patients and related equipment and laboratory, various wards, X-ray and pharmaceutical room, building, housing and canteen. The existing of WMPs in hospital showed that most hospitals have similar problems associated with the procedure of medical waste management. There is handling (including initial handling, storage, and transportation) problem that needs to be improved in order to decrease the potential of occupational risks. For example, the transportation of infectious waste in hospital should use elevator that delivers only product materials. The container that provides for general waste and infectious waste should have enough number of container in order to separate infectious waste from solid waste at source. If the infectious waste is contaminated with hazardous waste, it should be considered as hazardous waste and should be separate from infectious waste. In the case of infectious waste and hazardous waste, it should have secondary containment to protect any leak of waste from packaging during their collection, storage, and transportation.

Table 1. The sample of hospital in Chiang Mai, Thailand

Size & Type	Number of unit	Number of bed
1. Big, G	1	1,400
2. Medium, G	1	531
3. Medium, P	1	400
4. Medium, P	1	350
5. Small, P	1	180

Note: G = Government, P = Private

In some medium hospitals, there are recycling program that generate income for the hospital. This program should be expanded to other hospitals. In the process of storage, all hospitals have the color code and label system to identify the waste container. For example, black container is used for general or solid waste, yellow container is used for bottles and fluorescent lamps, and red container is used for infectious waste. The containers are made from plastic. In all hospitals, the treatment and disposal of medical waste, particularly, the infectious waste is a very critical step in the process of waste management. Most of this problem comes from the treatment technology. Presently, incinerator is the only accepted treatment option to treat infectious waste such as organic matter, tissue or amputated human body parts. A limitation of the incinerator is that air pollution problem during operation could not be controlled effectively. Moreover, the ash from incinerator has never been analyzed.

In some hospitals, the ash is buried in the hospital grounds. Furthermore, the operation and maintenance cost of incinerator is very high. Thus, the medium and small hospitals prefer to send their infectious waste for treatment in private incinerators or big hospital incinerators.

The treatment and disposal of hazardous wastes is also another problem of hospital waste due to the quantity of hazardous waste that needs not only special handling and storage but also specific treatment and disposal. In this case, the manual or standard operating procedure (SOP) should provide the important information for handling, storage, transportation, treatment and disposal of hazardous waste. In several hospitals, some liquid chemical waste can be recycled but others are discharged to the WWTP. Although all hospitals have their own WWTP (Table 3), some of them were experiencing problems with their WWTP due to the lack of experienced operators for maintenance and operation of the wastewater treatment system. Therefore, problems of wastewater quality were found during monitoring.

The quantity of medical waste generated in kilograms per day from different hospitals is shown in Table 4. It is found that the total quantity of waste generated from all medium and small hospitals was lower than the waste generated from big hospitals. Perhaps it is due to the big hospitals being equipped with modern facilities, and people prefer this kind of hospital for health care and treatment. It is noted that the solid waste or general waste in big (3,000 kg/day) and medium hospital ($<$ 830 kg/day) was higher than that in small hospital ($<$ 300 kg/day). The amount of hazardous waste in medium and big hospitals was in the same range (about 5 kg/day), but the quantity of this waste in small hospital is not available. In some hospitals, liquid hazardous waste was also generated. On comparison of unit (kg/day/bed) contribution of infectious waste, it is found that the big government university hospitals contribute the most. However, the quantity of medical waste can significantly be reduced if the hospital avoided the use of disposable medical care materials.

Table 2. Procedure of WMPs in hospitals

Procedure	Size & Type of Hospital				
	Big, Government	Medium, Government	Medium, Private	Medium, Private	Small, Private
I. Solid Waste (SW)					
– Source	– Buildings, housing & canteen	– Buildings, housing & canteen	– Buildings, housing & canteen	– Buildings, housing & canteen	– Buildings, housing & canteen
– Segregation	– At source & separate from IW	– At source & separate from IW	– At source & separate from IW	– At source & separate from IW	– At source & separate from IW
– Storage	– Black bag & Big bin	– Black bag & Big bin	– Black bag	– Black bag	– Black bag
– Transportation	– In (carriage & lift) & out (Truck)	– In (carriage & lift) & out (Truck)	– In (carriage & lift) & out (Truck)	– In (carriage & lift) & out (Truck)	– In (carriage & lift) & out (Truck)
– Treatment & Disposal	– Municipal Landfill	– Recycle & Disposal in Municipal landfill	– Municipal Landfill	– Municipal Landfill	– Municipal Landfill
II. Hazardous Waste(HW)					
– Source	– Wards, X-ray & pharmaceutical rooms	– Wards, X-ray & pharmaceutical rooms	– Wards, X-ray & pharmaceutical rooms	– Wards, X-ray & pharmaceutical rooms	– Wards, X-ray & pharmaceutical rooms
– Segregation	– Chemical Bottle, Battery, fluorescent lamp	– Chemical Bottle, Battery, fluorescent lamp	– Chemical Bottle, Battery, fluorescent lamp	– Chemical Bottle, Battery, fluorescent lamp	– Chemical Bottle, Battery, fluorescent lamp
– Storage	– Yellow Bin	– Identified container	– Identified Bin	– Identified container	– Bin
– Transportation	– In (carriage & lift) & out (Truck)	– In (carriage & lift) & out (Truck)	– In (carriage & lift) & out (Truck)	– In (carriage & lift) & out (Truck)	– In (carriage & lift) & out (Truck)
– Treatment & Disposal	– Private company	– Storage for disposal	– Storage for disposal	– Private company	– Municipal landfill
III. Infectious Waste (IW)					
– Source	– Patients & related equipment & lab.	– Patients & related equipment & lab.	– Patients & related equipment & lab.	– Patients & related equipment & lab.	– Patients & related equipment & lab.
– Segregation	– At source & separate from SW	– At source & separate from SW	– At source & separate from SW	– At source & separate from SW	– At source & separate from SW
– Storage	– Red bag & Bin	– Red bag & Bin	– Red bag & Bin	– Red bag & Bin	– Red bag & Bin
– Transportation	– In (carriage & lift) & out (Truck)	– In (carriage & lift) & out (Truck)	– In (carriage & lift) & out (Truck)	– In (carriage & lift) & out (Truck)	– In (carriage & lift) & out (Truck)
– Treatment & Disposal	– Autoclave, incinerator & Municipal landfill	– Autoclave, incinerator & Municipal landfill	– Autoclave, incinerator & Municipal landfill	– Autoclave, incinerator & Municipal landfill	– Autoclave, incinerator & Municipal landfill

Table 3. Wastewater treatment plant in hospitals

Size & Type	Type of WWTP	Main problem
1. Big, G	Activated Sludge	WWTP maintenance and operation
2. Medium, G	Activated Sludge & Oxidation Ditch	No expertise operator & WWTP maintenance and operation
3. Medium, P	Activated Sludge / Aerated Lagoon	Same
4. Medium, P	Activated Sludge	Same
5. Small, P	Activated Sludge	Same

Table 4. Quantity of medical waste generated in hospitals

Size & Type	Type of waste	Quantity generated (kg/day)
1. Big, Government	Infectious waste	0.97 kg/day/bed (1352.08)
	Solid waste	3,000
	Hazardous waste	4 – 5
2. Medium, Government	Infectious waste	0.53 kg/day/bed (279)
	Solid waste	830
	Hazardous waste	3 – 5
3. Medium, Private	Infectious waste	0.17 kg/day/bed (100)
	Solid waste	300
	Hazardous waste	–
4. Medium, Private	Infectious waste	–
	Solid waste	–
	Hazardous waste	6.7 l/day (liquid chemical waste)
5. Small, Private	Infectious waste	0.17 kg/day/bed (29.89)
	Solid waste	–
	Hazardous waste	–

Note: – not available

5 Conclusions

The WMPs in hospitals are drawn from the investigation as follow:

1) All hospitals sent general waste or solid waste to the municipal landfill.

2) The handling of medical waste should be properly implemented within hospitals and sent off site for disposal.

3) An adequate infrastructure for collection and transportation of the waste within and from each hospital to treatment facilities should be provided.

4) A pollution prevention plan should be prepared and hospital staff should be trained on the plan's implementation.

5) The incinerator should be improved and inspected to reduce the impact on air quality problem.

6) WWTP should be inspected regularly and be required to solve occurring problems.

7) A manual for handling hazardous waste should be prepared to cope with this waste in hospitals. The small quantity of hazardous waste should be accumulated until sufficient quantity is stored for transportation to the treatment and disposal facility that is located in the central part of Thailand, a long distance away, to avoid high cost of disposal.

8) The quantity of infectious waste in big hospitals is a higher rate (0.97 kg/day/bed) than that in medium hospital (0.17 – 0.53 kg/day/bed) and small hospital (0.17 kg/day/bed).

9) The standard operating procedure (SOP) that contains the outline of handling method, how collection, segregation, treatment and disposal as well as the regulation for segregation of infectious waste from hazardous waste should be clearly developed and operated.

10) Assigned agency should visit and inspect hospital's treatment facilities such as incinerators and wastewater treatment plants in order to improve the efficiency of these facilities and to solve problem when they occur.

11) Moreover, a website to promote efficient dissemination of information and to improve existing medical WMPs as well as to reduce environmental pollution and health hazards in the region should be done.

In conclusion, based upon the result of this study, it is recommended that the procedure of the

best WMPs in hospital should be provided as the SOP for implementation by the hospital staff. Moreover, the laws and regulations related to segregation of infectious waste from hazardous waste should be urgently imposed.

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