ANTICANCER RESEARCH *24*: 1753-1758 (2004)

Mitochondrial DNA Mutations in Light-associated Skin Tumors

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Abstract. *Mutations of mitochondrial DNA (mtDNA) have been proposed to be involved in carcinogenesis. In this study, we applied the polymerase chain reaction techniques to investigate the frequency of occurrence and proportion of mtDNA with length mutations (deletions and tandem duplications) in light-associated skin tumors (actinic keratosis, AK; basal cell carcinoma, BCC; and squamous cell carcinoma, SCC) in aged individuals. We demonstrated the existence of multiple mtDNA deletions and tandem duplications in tissues of AK, BCC, SCC and normal skin. We showed that the frequencies of occurrence of the 4,977 bp and 7,436 bp deletions and tandem duplications (200 bp and 260 bp) of mtDNA in light-associated skin tumors were not significantly different from those of sun-exposed normal skin ($p>0.05$), but higher than those of non-exposed normal skin ($p<0.05$). In addition, we found that the proportion of the 4,977 bp-deleted mtDNA in the skin of the same individual was also affected by skin pathologies. The proportion of 4,977 bp-deleted mtDNA in relatively rapid growing tumor cells in SCC was lower than that of normal skin cells. We suggest that the existence of these length mutations of mtDNA in normal, precancerous or cancerous human skin may be attributed to the stochastic effect of photo-damage. However, it is unclear whether the mutations of mtDNA have a direct bearing on carcinogenesis in skin. Future investigation is warranted to elucidate the causal relationship between mtDNA mutations and skin cancers and to address the pathophysiological role of mtDNA mutations in skin cancer development.*

Mitochondria possess their own genome, which is a 16,569 bp double-stranded circular DNA for the human cell. It encodes 13 polypeptides required for oxidative phosphorylation as well as the 12S and 16S rRNAs and 22 tRNAs necessary for protein synthesis in the mitochondria (1-3). There are 2-10 copies of mitochondrial DNA (mtDNA) in each mitochondrion, with up to 10^3 mitochondria per cell (1). Mitochondrial genome is more susceptible to DNA damage and consequently acquires mutations at a higher rate than does nuclear DNA (3). The higher susceptibility to damage of mtDNA may result from exposure to high levels of reactive oxygen species (ROS) produced during respiration, lack of protective histones and limited capacity for repair of DNA damage (3). Moreover, the fidelity of the DNA polymerase γ in mtDNA replication is lower than the other DNA polymerases in the nucleus (4).

Mitochondria have been implicated in the carcinogenic process because of their role in apoptosis and other aspects of tumor biology (5-7). The occurrence of mtDNA mutations in a variety of human cancers has recently been reported (5-18). The genetic changes include deletion, duplication, point mutation and microsatellite instability of mtDNA. It is thus of interest to investigate the possible role of mtDNA mutations in the process of carcinogenesis.

It is generally accepted that the ultraviolet radiation (UVR) of sunlight is the most important contributor to the development of actinic keratosis (AK) and non-melanoma skin cancers, including squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) (19). AK is thought to be the precursor of SCC and can be regarded as a clinical risk indicator of non-melanoma skin cancer (20). In this study, we used the polymerase chain reaction (PCR) techniques to investigate the frequency of occurrence and the proportion of deleted or duplicated mtDNA in these UV-associated skin pathologies, including AK, SCC and BCC.

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Key Words: Mitochondrial DNA deletion, tandem duplication, actinic keratosis, basal cell carcinoma, squamous cell carcinoma.

Materials and Methods

Patients and samples. We obtained 86 samples, including 10 SCC, 7 BCC, 16 AK, 31 sun-exposed normal skin tissues and 22 non-exposed skin tissues, from 77 patients in China Medical University Hospital, Taiwan. All the samples of SCC, BCC and AK were excised from the facial skin, except one metastatic SCC from the

Table I. *Nucleotide sequences of oligonuleotide primers for PCR amplification of mitochondrial DNA.*

Primer	Nucleotide position	Sequence(5'→3')
L1	(3,304 - 3,323)	AACATACCCATGGCCAACCT
L2	(8,150 - 8,169)	CCGGGGGTATACTACGGTCA
L3	(8,285 - 8,304)	CTCTAGAGCCCACTGTAAAG
L9	(8,344 - 8,363)	ACCAACACCTCTTTACAGTG
L336	(336 - 355)	AACACATCTCTGCCAAACCC
L7293	(7,293 - 7,316)	GCACTAATATTAATAATTTTCATG
L7538	(7,538 - 7,602)	TTAATGGCACATGCAGCAGCGCA
L7901	(7,901 - 7,920)	TGAACCTACGAGTACACCGA
L8251	(8,251 - 8,270)	GCCCGTATTTACCCTATAGC
L8356	(8,356 - 8,375)	TTACAGTGAAATGCCCCAAC
H1	(3,817 - 3,836)	GTGGAGACTAATGAGGACGG
H2	(13,650 - 13,631)	GGGGAAGCGAGGTTGACCTG
H3	(13,928 - 13,905)	CTAGGGTAGAATCCGAGTATGTTG
H335	(316 - 335)	CGAAGACCGGTGTCGTGAAT
H15877	(15,896 - 15,877)	TACAAGGACAGGCCCATTTG
H16208	(16,228 - 16,208)	GTTGAGGGTTGATTGCTGTAC
H16411	(16,430 - 16,411)	GCACTTTAGTTATAGGGCGT
H16422	(16,441 - 16,422)	TAGCACTCTTGTGCGGGATA

The sequences of the primers refer to the published nucleotide sequence of human mitochondrial genome (22).

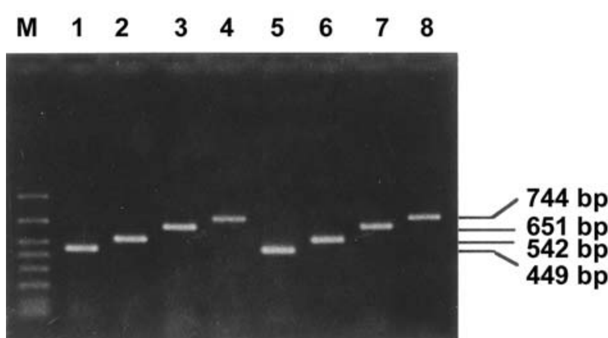

Figure 1. *Primer-shift PCR demonstrating the existence of the 7,436 bp deleted mtDNA in human skin tissues.*
Lanes 1 to 4 represent the PCR products of 449 bp, 542 bp, 651 bp and 744 bp that were amplified from the facial skin mtDNA of a 66-year-old male with the primers of L9-H16208, L8251-H16208, L9-H16411 and L8251-H16411, respectively (see Table I), under identical conditions. The change in sizes of the PCR products was consistent with the shift of length between primer sequences, indicating that the deletion was authentic and existed in the original tissues examined. Lanes 5 to 8 represent the PCR products of 449 bp, 542 bp, 651 bp and 744 bp that were amplified from the facial skin mtDNA of a 55-year-old female with the same primer pairs under identical conditions. M indicates the 100 bp ladder size marker.

right thigh. The ages of the patients were between 54 and 88, and the mean age was 70 years. Based on the medical history of the patients, the mean duration of the skin cancer was about 2 years. All the cases included in the study had no history or clinical manifestations of any specific mitochondrial myopathy or encephalomyopathy. Pathological examinations confirmed the diagnosis in all cancer patients.

Preparation of DNA. Samples were snap-frozen and stored at -70˚C. The total DNA mixture containing the mtDNA from the samples was prepared by the alkaline SDS lysis method according to the procedure described by Wallace *et al.* (21) with some modifications.

Synthesis of oligonucleotide primers. Oligonucleotide primers were chemically synthesized by Bio-Synthesis Inc. (Lewisville, TX, USA) according to the published sequence of human mitochondrial genome (22). The nucleotide sequences of the primers are shown in Table I.

Polymerase chain reaction (PCR). Each desired DNA fragment was amplified in a 50 µl reaction mixture containing 200 µM of each of the dNTPs, 1 unit of Taq DNA polymerase (Perkin-Elmer/Cetus), 0.4 µM of each primer, 50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 8.3). The reactions were carried out for 30 cycles in a Perkin-Elmer/Cetus DNA thermal cycler. The first cycle was done by 3 min denaturation at 94˚C, 3 min annealing at 55˚C and 1 min primer extension at 72˚C. The following PCR cycle parameters were as follows: denaturation at 94˚C for 40 sec, annealing at 56˚C for 40 sec and primer extension at 72˚C for 50 sec. In order to avoid the artifacts or false deletions generated by mis-pairing of primers to the mtDNA template, we checked the authenticity of each deletion by the primer-shift PCR method as previously described (23). The amplified PCR products were separated by electrophoresis on a 1.5% agarose gel at 100 volts for 40 min and the DNA bands were visualized under UV light transillumination after ethidium bromide (0.5 mg/ml) staining (Figure 1).

Semi-quantitative PCR method. For the determination of small proportions of the deleted mtDNA, we employed a quantitative PCR method as described previously (24). The extracted DNA from each of the specimens was serially diluted two-fold with distilled water. The range of dilution was usually between 2^0 to 2^{23}. The primers L1 and H1 were used for the amplification of a 533 bp DNA fragment from total mtDNA and the primers L2 and H2 were used for the amplification of a 524 bp PCR product from the 4,977 bp-deleted mtDNA. Amplified DNA fragments were separated by electrophoresis on 1.5% agarose gel at 100 volts for 40 min and were then detected fluorographically after staining with ethidium bromide (Figure 2). The gel pattern of DNA products was photographed under UV light transillumination. The proportion of 4,977 bp - deleted mtDNA was determined as the ratio of the highest dilution fold that allowed the 524 bp PCR product to be visible on the gel to that which allowed the 533 bp PCR product to be visibly amplified from total mtDNA under identical conditions (24).

Detection of the tandem duplications in the D-loop of mtDNA. Using the back-to-back primer pair L336-H335 as described by Brockington *et al.* (25), we detected tandem duplications following the PCR protocol as mentioned above. There should be no PCR products obtained in wild-type mtDNA, but we could obtain DNA fragments with specific sizes if there were duplications in the D-loop region of mtDNA (Figure 3).

Statistical analysis. Comparison was made between the frequency of occurrence of mtDNA mutations (4,977 bp, 7,436 bp deletions

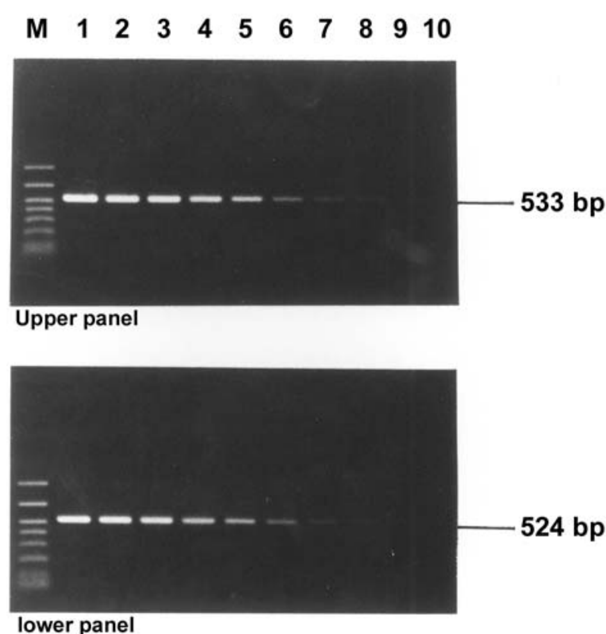

Figure 2. *The semi-quantitative PCR method to determine the proportion of 4,977 bp-deleted mtDNA.*
The proportion of 4,977 bp-deleted mtDNA in the facial skin of a 76-year-old female was determined by a serial dilution method. Lanes 1 to 11 in the upper panel represent the PCR products amplified from total mtDNA which was serially diluted to 2^{11}, 2^{12}, 2^{13}, 2^{14}, 2^{15}, 2^{16}, 2^{17}, 2^{18}, 2^{19} and 2^{20}-fold, respectively, by using primer L1 (3304-3323) and H1 (3817-3836) under conditions described in Materials and Methods. Lanes 1 to 11 in the lower panel represent the PCR products specifically amplified from 4,977 bp-deleted mtDNA which was serially diluted to 2^4, 2^5, 2^6, 2^7, 2^8, 2^9, 2^{10}, 2^{11}, 2^{12} and 2^{13}-fold, respectively, by using the primers L2 (8150-8166) and (13631-13650) under identical conditions. The proportion of the 4,977 bp-deleted mtDNA in this case was 1.6% (4,977 bp-deleted mtDNA/total mtDNA = $2^{11}/2^{17}$). M indicates the 100 bp ladder size marker.

and tandem duplications) in skin with light-associated pathologies (AK, SCC and BCC) and that of the normal skin (exposed and non- exposed). The difference in the frequency of occurrence between the two groups was analyzed by the Chi-square test. A p value below 0.05 was considered statistically significant.

Results

The frequency of length mutations in mtDNAs from various skin tumors. The frequencies of occurrence of the 4,977 bp and 7,436 bp deletions of mtDNA in skin biopsies were determined by PCR using the primers L2 and H2 and the primers L8251 and H16208, respectively. The PCR products were confirmed by primer-shift PCR (Figure 1). The frequencies of occurrence of the 4,977 bp and 7,436 bp deletions of mtDNA in various skin pathologies are summarized in Table II. The frequencies of occurrence of 4,977 bp-deleted mtDNA in normal non-exposed skin, normal exposed skin, AK, SCC and BCC were 31.8%, 61.3%, 81.3%, 70.0% and 71.4%, respectively. The frequencies of occurrence of the 7,436 bp deletion of mtDNA in normal non-exposed skin, normal exposed skin, AK, SCC and BCC were 0%, 19.4%, 25.0%, 0% and 42.9%, respectively. In addition, we found that the frequency of occurrence of the 4,977 bp deletion of mtDNA in sun-exposed normal skin was significantly higher than that in AK, SCC and BCC. However, the frequencies of occurrence of the 4,977 bp deletion of mtDNA in AK, SCC and BCC were significantly higher than that of the non-exposed normal skin ($p<0.05$). Moreover, the frequencies of occurrence of both the 4,977 bp and 7,436 bp deletions of

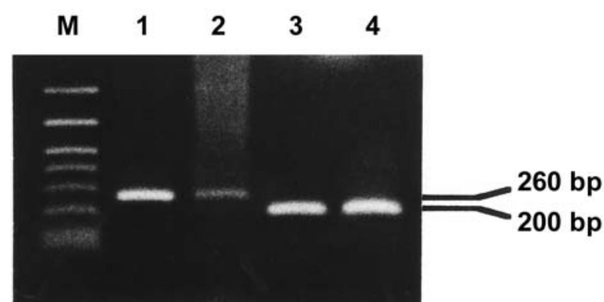

Figure 3. *PCR amplification of the 200 bp and 260 bp tandem duplications in the D-loop of mtDNA from human skin tissues.*
The primers L336-H335 were used to amplify the target sequence in the D-loop region of human mtDNA. The primers were so designed (back to back) that no PCR product could be generated from normal mtDNA and only those mtDNA molecules containing the putative tandem duplications would be amplified to give rise to PCR products with distinct sizes. Lanes 1 and 2 represent the 260 bp PCR products amplified from two patients aged 55 and 66 years old, respectively. Lanes 3 and 4 reveal the 200 bp PCR products amplified from two patients aged 68 and 76 years old, respectively. M indicates the 100 bp ladder size marker.

Table II. *Incidences of the length mutations of mitochondrial DNA in various skin pathologies.*

Diagnosis (No. of samples examined)	D4977 No. (%)	D7436 No. (%)	DUP No. (%)
NES (31)	19 (61.3)	6 (19.4)	9 (29.0)
NNES (22)	7 (31.8)	0 (0)	1 (4.5)
AK (16)	13 (81.3)	4 (25.0)	5 (31.3)
SCC (10)	7 (70.0)	0 (0)	1 (10.0)
BCC (7)	5 (71.4)	3 (42.9)	4 (57.1)*

NES, normal exposed skin; NNES, normal non-exposed skin; AK, actinic keratosis; SCC, squamous cell carcinoma; BCC, basal cell carcinoma; D4977, 4,977 bp-deleted mtDNA; D7436, 7,436 bp-deleted mtDNA; and DUP, tandem duplications in the D-loop region of mtDNA. All the patients with tandem duplication(s) were found to harbour the 200 bp duplication, but there were 2 cases (*) bearing the 260 bp duplication.

Table III. *Proportion of the 4,977 bp-deleted mt DNA in various skin pathologies.*

Diagnosis	No. of samples	Max (%)	Mean (%)	S.D. (%)
NES	13	12.5	1.31	3.49
NNES	7	1.56	0.18	0.39
AK	10	3.13	0.56	1.07
SCC	7	3.13	0.4	0.97
BCC	5	1.56	0.57	0.7

Max, maximum value of the proportion of 4,977 bp-deleted mtDNA in each group; Mean, mean value of the proportion of 4,977 bp-deleted mtDNA in each group; S.D., standard deviation; NES, normal exposed skin; NNES, normal non-exposed skin; AK, actinic keratosis; SCC, squamous cell carcinoma; and BCC, basal cell carcinoma.

Table IV. *Comparison of the proportion of the 4,977 bp-deleted mtDNA in various skin pathologies of the same individuals.*

Case No.	Age/Sex	NES	NNES	AK	SCC
1	64/M	2^{-8}	0	2^{-8}	NA
2	66/M	NA	2^{-8}	NA	2^{-12}
3	69/M	2^{-8}	0	2^{-8}	NA
4	75/M	2^{-8}	2^{-9}	NA	2^{-9}
5	75/M	2^{-4}	2^{-6}	2^{-5}	NA
6	80/M	2^{-3}	NA	NA	2^{-10}
7	86/M	2^{-3}	2^{-8}	2^{-6}	2^{-5}
8	88/M	2^{-6}	0	NA	2^{-8}

NES : normal sun-exposed skin; NNES : normal non-exposed skin; AK: actionic keratosis; SCC: squamous cell carcinoma; NA: not available.

mtDNA in the exposed skin were also significantly higher than those in the non-exposed skin ($p<0.05$).

The tandem duplications in the D-loop of mtDNA in various skin tumors. Using PCR and the primers L336 and H335, we detected 200 bp and 260 bp tandem duplications in the D-loop region of mtDNA in some of the skin samples. The frequencies of occurrence of the tandem duplications of mtDNA in normal non-exposed skin, normal exposed skin, AK, SCC and BCC were 4.5%, 29.0%, 31.3%, 10.0% and 57.1%, respectively. The most frequently seen was the 200 bp tandem duplication, but two of the BCC samples were found to harbor the 260 bp tandem duplication. The frequencies of occurrence of the two tandem duplications of mtDNA in various skin pathologies are shown in Table II. The tandem duplications may occur alone or in combination with 4,977 bp deletion of mtDNA in some skin samples.

Quantitative determination of 4,977 bp-deleted mtDNA in various skin tumors. Using a semi-quantitative PCR method, we determined the proportion of 4,977 bp-deleted mtDNA among the 35 skin samples that had been confirmed to harbor the deletion. The average proportions of the 4,977 bp-deleted mtDNA in non-exposed normal skin, exposed normal skin, AK, SCC and BCC were 0.18%, 1.31%, 0.56%, 0.40% and 0.57%, respectively (Table III). Exposed normal skin usually harbored the highest amount of the deleted mtDNA, SCC or BCC had less deleted mtDNA, while the non-exposed skin harbored the lowest amount of deleted mtDNA. The maximum proportion of 4,977 bp-deleted mtDNA was 12.5% in one of the exposed normal skin samples. All the samples disclosed heteroplasmy of mtDNA in various skin pathologies.

Comparison of the levels of 4,977 bp-deleted mtDNA in various skin tumors of the same patients with SCC. We obtained multiple biopsies from the same individuals in 8 SCC cases (Table IV). We found that the frequency of occurrence of the 4,977 bp deletion of mtDNA had regional and pathological variations in the same individuals. The 4,977 bp deletion of mtDNA was found to occur either in the exposed skin alone or in exposed and non-exposed skin simultaneously. In this study, the 4,977 bp deletion of mtDNA was never found to occur in the non-exposed skin but was absent in sun-exposed skin of the same individuals. If the mtDNA deletion occurred simultaneously in multiple sites, the proportion of 4,977 bp-deleted mtDNA was found to be in the following order: exposed normal skin >AK > SCC > non-exposed normal skin. The exposed normal skin usually harbored the highest level of 4,977 bp-deleted mtDNA among various regions and pathologies of skin. The proportion of 4,977 bp-deleted mtDNA in relatively rapid growing tumor cells in SCC was lower than that in exposed normal skin. The proportion of 4,977 bp-deleted mtDNA in exposed normal skin could reach as high as 12.5%.

Discussion

In this study, the specimens for mtDNA analysis were all taken from elderly subjects, with a mean age of 70. The frequencies of occurrence of mtDNA deletions and tandem duplications in precancerous (AK) or cancerous skin tissues (SCC and BCC) were not significantly different from those of the exposed normal skin. Moreover, the frequencies of occurrence of these mtDNA mutations in the exposed skin were significantly higher than those in the non-exposed skin. These results and our previous findings that the frequencies of mtDNA deletions increase with age suggest that the occurrence of mtDNA deletions in skin is closely related to stochastic events, such as ultraviolet irradiation (26-28).

We also demonstrated that the maximum and average proportions of 4,977 bp-deleted mtDNA were highest in exposed normal skin, intermediate in AK, BCC, or SCC, and

lowest in the non-exposed normal skin (Table III). Moreover, in the study of multiple skin pathologies from the same SCC patients, we also found that the exposed normal skin usually harbored the highest level of 4,977 bp-deleted mtDNA among various light-associated skin abnormalities. The proportion of 4,977 bp-deleted mtDNA in rapidly growing tumors in SCC was lower than that in exposed normal skin (Table III). Our findings that BCCs harbored higher levels of mtDNA with 4,977 bp deletion than did SCCs are consistent with the report of Durham *et al.* (18). Lee *et al.* (16) proposed possible mechanisms to explain this phenomenon. Firstly, mutant mtDNA is diluted in the tumor tissue by the high mitotic activity of tumor cells. Secondly, a bioenergetic restraint may limit the growth of tumor cells. Recently, Coller *et al.* (29) and Jones *et al.* (30) also proposed that somatic mtDNA mutations accumulated in tumors could be the result of random genetic drift. Moreover, Chinnery *et al.* (31) put forward a hypothesis that random genetic drift is a common mechanism involved in human aging, cancer and mitochondrial disease. Ray *et al.* (32) demonstrated that mtDNA deletion is an ubiquitous marker of human skin exposed to UV irradiation. Moreover, Birch-Machin *et al.* (33) demonstrated that mtDNA deletions reflect photoaging rather than chronological aging of skin. These reports and our findings suggest that deletions and tandem duplications of mtDNA occurring in human skin cancers may well be attributed to the stochastic effect of photoaging and may not be directly involved in cutaneous carcinogenesis.

On the other hand, Koch *et al.* (34) demonstrated that repetitive UVA irradiation induced 4,977 bp deletion of mtDNA in human keratinocytes. UV irradiation is known to produce DNA damage in the tumor suppressor genes or oncogenes, *e.g. p53* and *Fas*, which may in turn lead to the development of skin cancers (35, 36). Although the mtDNA fragment inserted into a c-myc oncogene in some tumor cells has been reported (37), it can be argued that the insertion of mtDNA fragments into the nuclear genome is just a rare, random and accidental event. Nevertheless, the high frequency of mtDNA mutation and mitochondrial genome instability, in combination with the high sensitivity of PCR-based assays, may serve as a potential new biomarker for early detection of skin cancers (17).

In conclusion, we demonstrated the existence of multiple mtDNA deletions and tandem duplications in normal, AK, BCC and SCC of human skin. We suggest that the occurrence of these length mutations of mtDNA in normal, precancerous or cancerous human skin may be attributed to the stochastic effect of photo-damage. However, it remains unclear as to how mutations of mtDNA are involved in cutaneous carcinogenesis. Future investigations are needed to elucidate the causal relationship between mtDNA mutations and skin cancers and to address the role of mtDNA mutation in the initiation and progression of skin cancers.

Aknowledgements

This work was supported by grants from the National Science Council (NSC90-2320-B010-079) and the National Health Research Institute of Taiwan, (NHRI-EX91-9020BN). the Republic of China.

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Received February 19, 2004
Revised March 12, 2004
Accepted April 20, 2004