附件：封面格式

行政院國家科學委員會補助專題研究計畫成果報告

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※ 食道癌遺傳因子喪失與環境暴露之關係研究 ※

※ The Relation of Environment Exposure and Allelic Loss in ※

※ Esophageal Cancer ※

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計畫主持人：李章銘

共同主持人：

計畫參與人員：黃淑靖

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：臺大醫院外科

中　華　民　國九十一年　十 月三十一日

計畫編號: NSC90-2314-B002-431
計畫名稱:食道癌遺傳因子喪失與環境暴露之關係研究
The Relation of Environment Exposure and Allelic Loss in Esophageal Cancer
執行期限:90 年 8 月 1 日至 91 年 7 月 31 日
主持人: 李章銘醫師 email:jangming@ha.mc.ntu.edu.tw
執行機構及單位名稱: 台大醫院外科

中文摘要

關鍵詞：Esophageal cancer, p53, mutation
食道癌在台灣，仍是一威脅國民健康的重要疾病。它的發生與多種環境因子的暴露有關。在先前的研究中，我們發現台灣的食道癌，與抽煙，喝酒與嚼食檳榔等環境毒物的暴露有關。隨著這些環境毒物暴露劑量的增加，食道癌發生的危險性，亦隨之呈等比的增加。這三個因子，對彼此的致癌作用，亦有明顯的互相增強的現象。而在過去我們以顯微割離(Laser Captrue Microdissection, LCM)的技術，並運用位於第十七，與十八對染色體的一系列微小星體標記(microsatellite markers)，以 PCR 反應，比較正常與腫瘤 DNA 的產物。我們發現了食道癌腫瘤在十七，十八對染色體上，有多處遺傳體喪失。這些遺傳體缺失的位置，有些與目前我們所知的腫瘤抑制基因的位置相吻合。有些則尚未有文獻提及。尤其有趣的是，我們發覺在嚼檳榔者，較不易在 17p12 出現遺傳體喪失(P<0.05，與非嚼檳榔者比較) 。此結果，顯示了在嚼檳榔者與非嚼檳榔者，可能是透過不同的機轉而產生食道癌。這個位置恰好是腫瘤抑制基因 p53 所在的遺傳位置。為了更進一步釐清食道癌與這些環境毒物有關的致癌機轉， 我們以顯微割離(Laser Captrue Microdissection, LCM)的技術萃取腫瘤組織之 DNA，以 PCR 反應及 DNA 定序，探討環境暴露因子對 p53 突變之影響。到目前為止我共完成了 Exon 4、6、9 的 DNA 定序，我們發現不同的環境暴露背景，可以造成不同的 p53 突變型式與位置，這些位置對 p53 功能的影響，還有待進一步研究。

Abstract

Esophageal cancer is still an important disease in Taiwan. Various environmental exposures have been associated with the risk of esophageal cancer. In the previous study, we have found that the risk of esophageal cancer can be significantly increased by the habits of cigarette smoking, alcohol drinking and areca chewing. The risk was positively correlated with the exposure dosage of these factors. These three factors also act synergistically to enhance the individual susceptibility of esophageal cancer. In the recent project, using the technique of laser capture microdissection (LCM) and polymerase-chain-reaction-based polymorphic markers located on chromosomes 17 and 18, we have detected multiple allelic deletions (LOH) on these areas. Some of the LOHs are compatible with the locations of the known tumor suppressor genes(TSG), i.e. p53 [17p13.1], NF1[17q11.2], BRCA1[17q21], SMAD4[18q21], DCC [18q21.3]. However, some of them are not, indicating more potential tumor suppressor genes are yet to be found in these areas. We found that the areca chewers had a significantly higher tendency for retention of heterozygocity in 17p12 ($p<0.05$, as compared to non-chewers). It implies that the environmental exposure may determine the mutation or expression in specific tumor suppressor genes due to the different mechanisms of the chemicals-associated carcinogenesis. To further clarify this, we proposed the study to investigate the association of p53 mutation and environmental exposure of the patients. We used the technique of LCM to obtain the tumor genomic DNA. P53 sequencing was

performed after PCR amplification using primers locating on exons 4-9 of p53 gene. At the present time, we have finish the work of sequencing on exons 4, 6 and 9 of p53. Form the preliminary data, we found that mutation pattern is affected by the environmental exposure of the patients.

Text

The esophageal cancer is still a dismal disease despite of the modern treatment modalities. The annual incidence of esophageal cancer in Taiwan is 6.93 per 100,000 populations (1), which is similar to that of less than 10 per 100,000 populations in most Western countries (2 , 3). However, some areas in North China are suffering from an extraordinarily high prevalence of esophageal cancer. Take Linxian for example, the annual incidence in this place exceeds 100 per 100,000 populations, which is the first cause of death locally (2 , 3). The risk of esophageal cancer has been associated with various environmental and genetic factors. In the industrialized countries, cigarette smoking and alcohol drinking are the most important environmental factors for developing esophageal cancer (4). Dietary effects, such as malnutrition, inadequate intake of fruits and fresh vegetables, or frequent consumption of pickled vegetables were also found to be important in some high-risk areas of China (2, 5).

Previously we have found that the risk of esophageal squamous cell carcinoma was obviously increased by the three environmental factors, i.e., cigarette smoking, alcohol drinking and areca chewing (6). The risk was positively correlated to the dosage of exposure to these substances (Table 1). The OR for cigarette smokers who consumed 30 pack-year of tobacco or less was 5.39 (95% CI: 2.73-10.65), while the OR rose to 14.62 (95% CI: 7.26-29.45) for heavy smokers who smoked more than 30 pack-years of tobacco. Likewise, the ORs for light and heavy alcohol drinkers (< 157 gram-years vs. > 157 gram-years of alcohol) were 2.57 (95% CI: 1.24-5.34) and 10.93 (95% CI: 6.20-19.15), respectively. The risk of esophageal cancer also increased with lifetime areca consumption, with an OR of 10.95 (95% CI: 4.66-25.26) for those who chewed 494 betel-years or less and 40.53 (95% CI: 11.51-142.63) for those who chewed more than 494 betel-years. We also found that these factors exerted a multiplicative effect on the risk of esophageal cancer (Table 2). In those exposed to only one of these factors, the OR for esophageal cancer was 1.4 (95%CI: 0.7-2.9). For those exposed to two factors, the OR was 4.4 (95% CI: 2.2-8.8). Those exposed to three of these factors had an OR up to 18.5 (95% CI: 8.9-38.7) (6). This dose-response relationship for alcohol and tobacco usage was consistent with previous studies (7, 8, 9,10). Cheng et al. (8) showed that the risk of esophageal cancer in Hong Kong was independently enhanced by smoking and drinking. Smokers consuming tobacco less than 15 gm per day had a three-fold higher risk for esophageal cancer compared to non-smokers, while heavy smokers consuming more than 25 gm of tobacco per day had more than a 10 fold higher risk for developing esophageal cancer. Individuals who drank less than 200 gm of alcohol per week had a 1.4 fold higher risk for esophageal cancer compared to non-alcohol drinkers; this risk increased up to a 14 fold higher risk for those who drank more than 400 gm of alcohol per week (8). A similar dose-response relation was also found in studies from Western countries (7,9,10). These two factors can strengthen the effect of each other when combined (5 ,6, 11,12,). Heavy smokers and heavy drinkers in Italy have a 7-fold higher risk of cancer, while individuals who simultaneously consume alcohol and tobacco have a 17-fold higher risk (11). Areca chewing is popular in Southeast Asia and was found to be closely related to oral cancer in Taiwan (13). Previously, others and we demonstrated that the habit of consuming areca quid significantly increases the risk of esophageal cancer (14 ,6). Areca chewing can act synergistically with cigarette smoking and alcohol drinking for developing esophageal cancer (6) (Table 2). The manner of areca nut chewing is unique in Taiwan and different from that of Indian, where the consumption is usually mixed with tobacco (14). People in Taiwan usually mix the betel nut with Piper betel inflorescence (sometimes substituted by betel leaf) and lime paste, without

tobacco . Our study has provided more direct evidence for the areca-associated risk of esophageal cancer. A high content of safrole in Piper betel (15 mg/gm) is believed to be an important carcinogen that accompanies areca nut chewing (15 and). Safrole promotes the formation of safrole-DNA adduct via sulfonation, conjugating an unstable sulfuric acid ester to the nucleotide (16). The extract of areca can also induce oxidative damage to DNA through the generation of hydrogen peroxide (8-hydroxy-2'- deoxyguanosine, 8-OH-dG). (17) The main potential carcinogens present in tobacco smoke include polyaromatic hydrocarbons (PAH), heterocyclic hydrocarbons, N-nitrosamines, aromatic amines, aldehydes, volatile carcinogens, inorganic compounds, and radioactive elements (18). Exposure to these pollutants can increase the risk of DNA damage, as evidenced by the non-linear dose-response correlation between smoking and PAH-DNA adduct in lekocytes of smokers (19). In vitro data also showed that products from cigarette smokes can induce direct DNA damage, and inhibit DNA repair following gamma- irradiation in human lymphoid cells (20). Excessive alcohol consumption can also lead to DNA damage, mainly through the production of reactive oxygen species of oxygen radicals, lipid peroxidation products, or acetaldehyde (21). In rats, chronic exposure to ethanol increases free radical generation in the liver and induces the activity of xenobiotic metabolizing enzymes (CYP 2E1). The frequency of single-strand breaks of DNA also increase after chronic alcohol exposure (22). In humans, long-term alcohol intake induces genetic alterations evidenced by chromosome aberrations and the presence of micronuclei in lymphocytes. This damaging effect is in synergy with tobacco smoke and can be reversed by abstinence (23). In primate tissues, especially those of the gastrointestinal and urogenital organs, ethanol co-exposure can lead to striking increase in DNA adducts formation induced by the carcinogen, N-nitrosodimethylamine (NDMA) (24). Previously, using the technique of laser capture microdissection (LCM) and polymerase-chain-reaction-based polymorphic markers located on chromosomes 17 and 18, we have detected multiple allelic deletions (LOH) on these areas (Table 3). Some of the LOH are compatible with the locations of the known tumor suppressor genes (p53 [17p13.1] : 41.2% LOH; NF1[17q11.2]: 26.7 % LOH; BRCA1[17q21]: 37.1% LOH; SMAD4[18q21]: 47.1%LOH; DCC [18q21.3]: 62.5%LOH). However, some of them are not, indicating more potential tumor suppressor genes are yet to be found in these areas. We also found that the patterns of allelic deletion were influenced by the habits of cigarette smoking, alcohol drinking and areca chewing. The allele located on 17q21 was preferentially deleted in cigarette smokers ($p=0.57$ as compared to the non-smokers) (Table 4). The allele on 17p12 was more frequently deleted in the areca chewers ($p=0.014$ as compared to the non-chewers)(Table 4). The allele on 18p11.2 was more frequently deleted in alcohol drinkers ($p=0.044$ as compared to the non-drinkers)(Table 4). In contrast, the areca chewers had a significantly higher tendency for retention of heterozygocity in 17p12 ($p<0.05$, as compared to non-chewers) (Table 4). 17p12 is near the location of p53 (25). It was found that p53 mutation is more often present in the cigarette smokers and alcohol drinkers (26). It was also found that the cigarette or areca-related oral squamous cel carcinoma were frequently accompanied with the overexpression of p53 (27). Other's and our data imply that the environmental exposure may determine the mutation or expression in specific tumor suppressor genes due to the different mechanisms of the chemicals-associated carcinogenesis. To further clarify this, we proposed the study to investigate the association of p53 mutation and environmental exposure of the patients. We used the technique of LCM to obtain the tumor genomic DNA. P53 sequencing was performed after PCR amplification using primers locating on exons 4-9 of p53 gene. At the present time, we have finish the work of sequencing on exons 4, 6 and 9 of p53. From the preliminary data, we found that mutation pattern is affected by the environmental exposure of the patients.

Materials and Methods

Study Population

This project is part of an ongoing cooperative study aimed at understanding the causes esophageal cancer in Taiwan. It will include incident cases of esophageal squamous cell carcinoma diagnosed at National Taiwan University Hospital from 1996 to 2003. The patients will be randomly selected from this esophageal cancer cohort. Institutional review board-approved informed consent will be obtained from each patient prior to tissue acquisition. Their background of environmental exposure including smoking, drinking and other possible risk factors for esophageal cancer will be collected by structured questionnaire. Peripheral blood of the patients will be drawn before surgery or chemoirradiation. All specimens will be maintained at -80oC until subsequent analysis. Blood will also be drawn from a control group of normal healthy persons matched for age (with a difference of less than 5 years) and sex, who comes to National Taiwan University Hospital for routine medical examination.

DNA Extraction from the Blood

Genomic DNA will be prepared from the leukocytes with serial extraction by phenol and chloroform, and then ethanol precipitation. The content of DNA of each sample will be analyzed by spectrophotometry, and about 1 μg of DNA will be taken from each sample for PCR amplification.

Laser Capture Microdissection. (LCM)

Laser capture microdissection, i.e. LCM will be performed to ensure the purity of tumor sample. Usually, more than 90% of purity of the tumor cells can be attained with this technique. LCM is performed on the immunostained slides using a PixCell laser capture microscope according to the technical quidance of the manufacturer (Arcturus Engineering, Mountain View, CA). Briefly, the stained, dehydrated tissue section is overlaid with a thermoplastic film mounted on an optically transparent cap. The visually selected areas (tumor cells) are bound to the membrane by short, energy laser pulses resulting in focal melting of the polymer, The cells will be immersed in 50-100 μl Tris buffer, pH 8.0, containing 0.5 mmol/L EDTA and 400 μl /ml proteinase K , and digested for 24 hours at 55^{o}C. After digestion, the enzyme is heat-inactivated and the extract is used directly for alleotyping PCR (LOH detection). Multiple efforts will be made to optimize LCM conditions, including to improve capture efficiency by overnight pretreatment of slide with xylene, and to ensure successful PCR amplification by avoiding fragmentation of DNA in captured tissue using a modified protocol of tissue fixation.

Direct sequence of amplified DNA

For the suspected TGS with high prevalence of LOH around its location, a direct sequencing strategy will be performed for the PCR products using adequate primers according to the known sequences of TGS in the GenBank (1). The double strand TGS template will be purified using Qiaquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and sequenced by the Sanger dideoxy chain terminator method, with dye-labeled dideoxy terminator chemistry. DNA samples will be analyzed in a Perkin Elmer 310 automated DNA sequencer (Applied Biosystems, AC, USA). To avoid mistake during the Taq polymerase amplification process, at least three different PCRs will be performed and their products sequenced.

Results and discussions

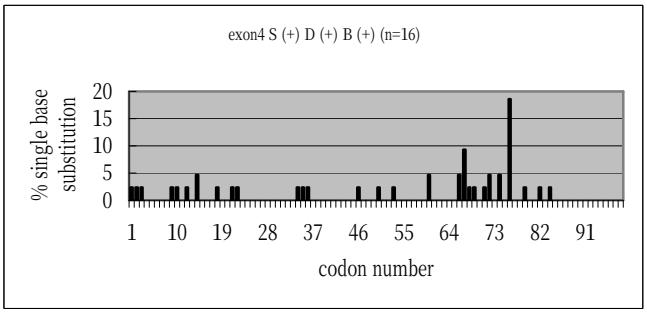
Mutation of p53 in exon 4 of esophageal cancer

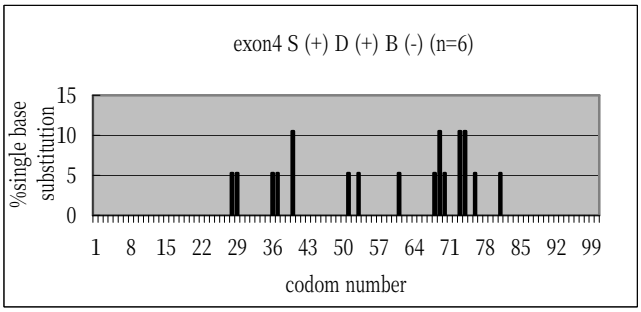
Up to now, we have analyzed the tumor DNA obtained by laser capture microdissection (LCM). Totally, 41 tumor specimen were under analysis. At the present time we have finish the sequencing of p53 on exon 4 , which was analyzed by the Sanger dideoxy chain terminator method, with dye-labeled dideoxy terminator chemistry. The process was carried in a Perkin Elmer 310 automated DNA sequencer (Applied Biosystems, AC, USA). The detailed of p53 mutation and the

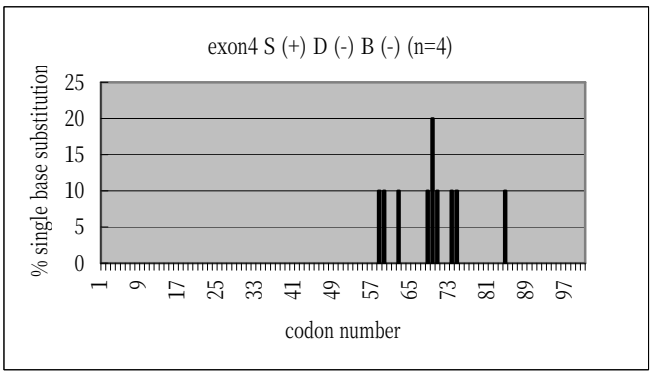
association with environmental exposure is shown in Fig 1-2.

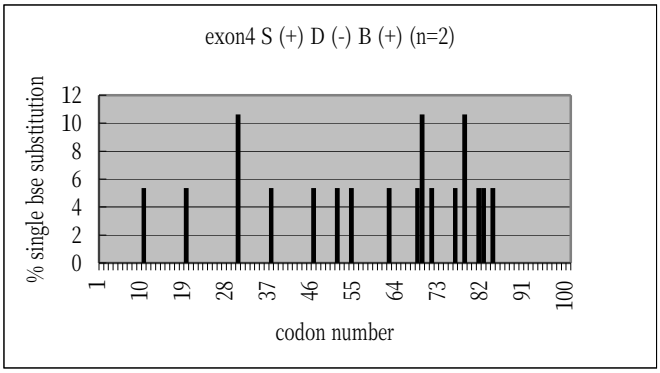
Detection of the mutation spectrum of TP53 has been used as an indicator for carcinogenic effects of the environmental carcinogenes in defined types of cancer. (28-9) TP53 is frequently mutated in human tumors. (30) In 40-50% of all esophageal cancers TP53 mutations have been found, most frequently missense mutations, but also nonsense mutations, deletions and insertions. (31-3) They are clustered in exons 5-8 that contain the DNA binding domain of *TP53*. Most mutations alter the p53 protein structure and lead to loss of its tumor suppressor function. (29, 32). Our data demonstrated confirm the concept that exposure to different carcinogen can induce different pattern of p53 mutation and lead to esophageal carcinogenesis finally.

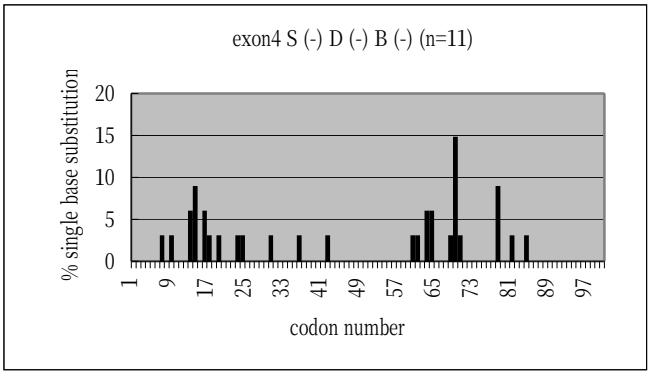
Exon 4





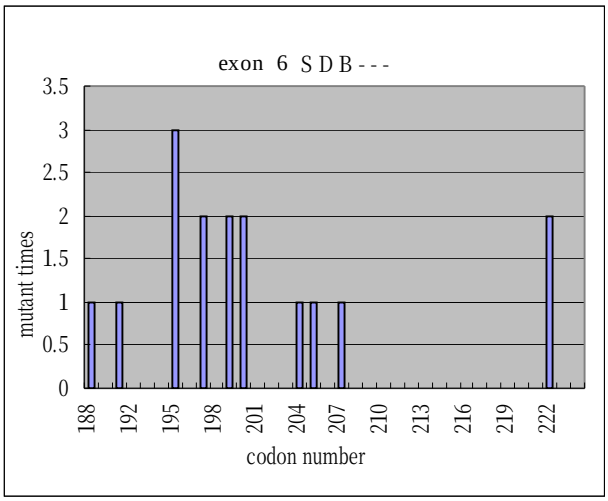


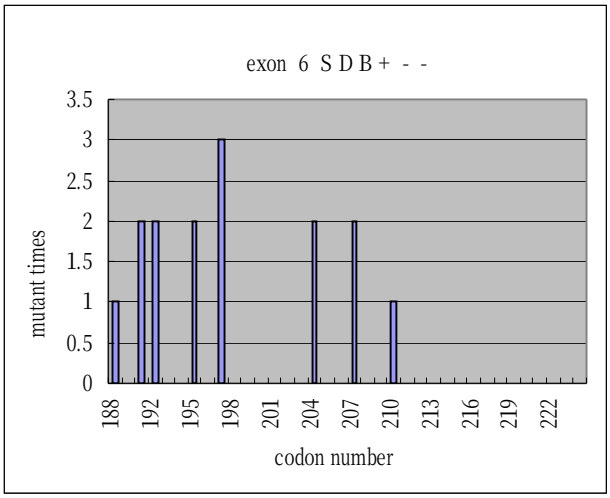


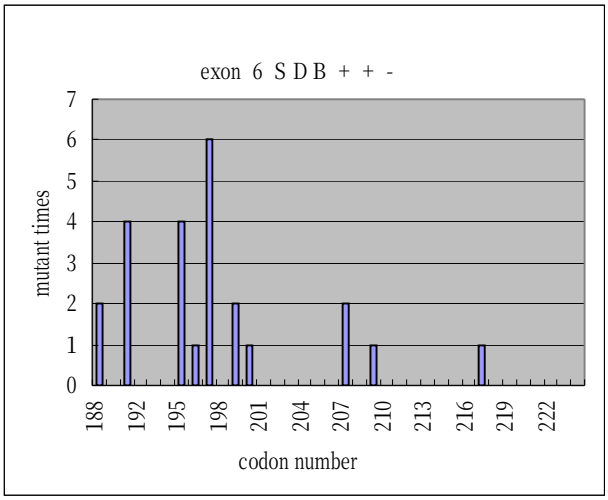


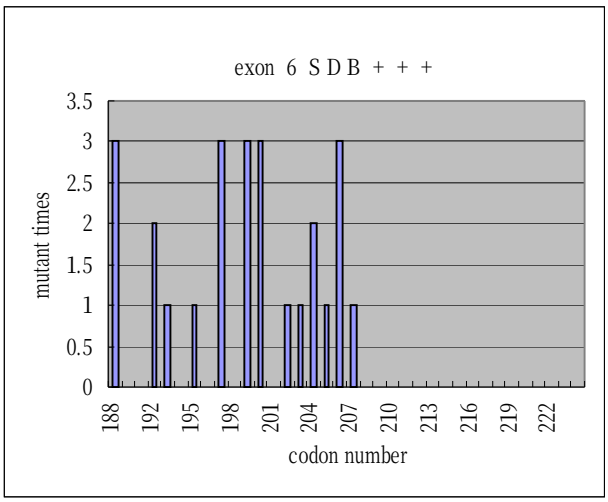
S: smoke; D: drinking; B: betal chewing; codon number: starting from the codon of exon 4 of p53.

Exons 5



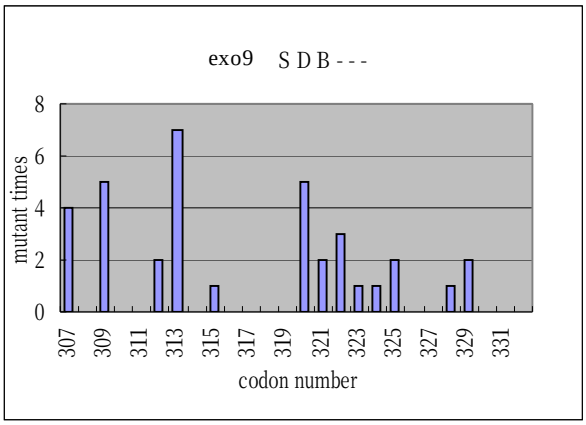






S: smoke; D: drinking; B: betal chewing; codon number: starting from the codon of exon 4 of p53.

Exon 9


exo9 S D B - - -
mutant times
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2
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6
8
307
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311
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315
317
319
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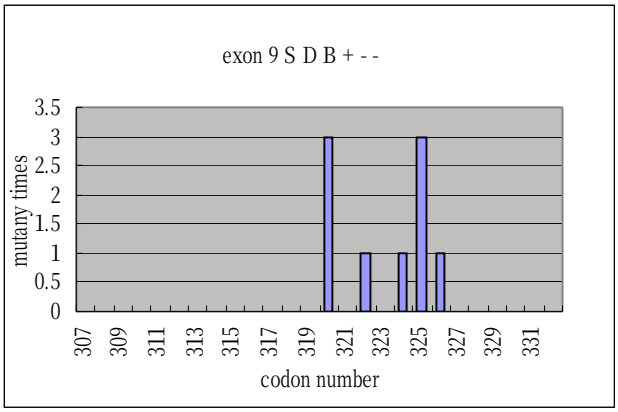
323
325
327
329
331
codon number


exon 9 S D B + - -
mutany times
0
0.5
1
1.5
2
2.5
3
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319
321
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327
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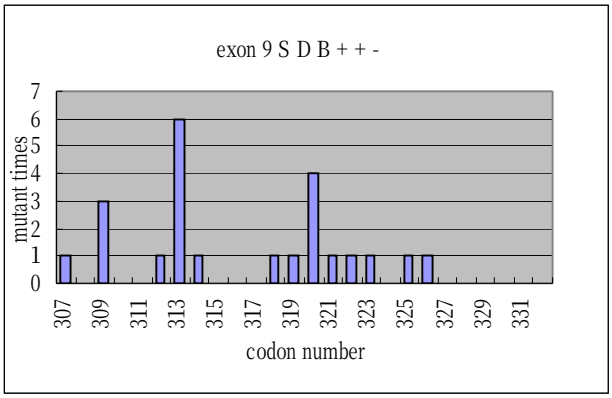
331
codon number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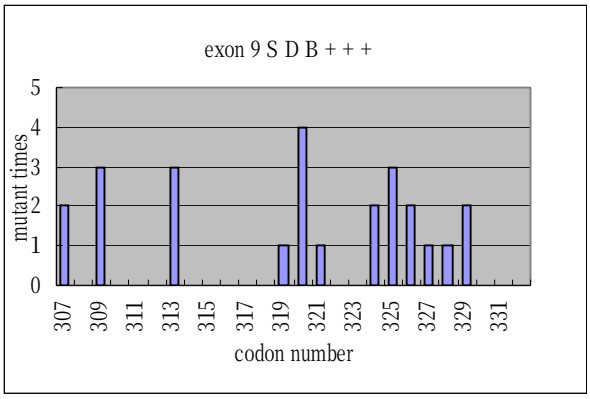
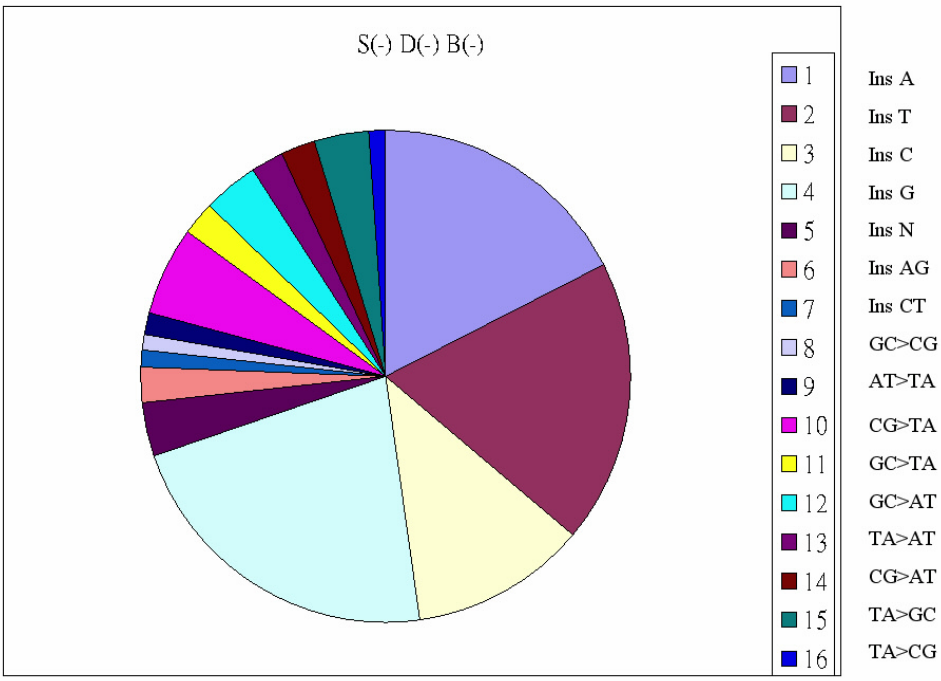
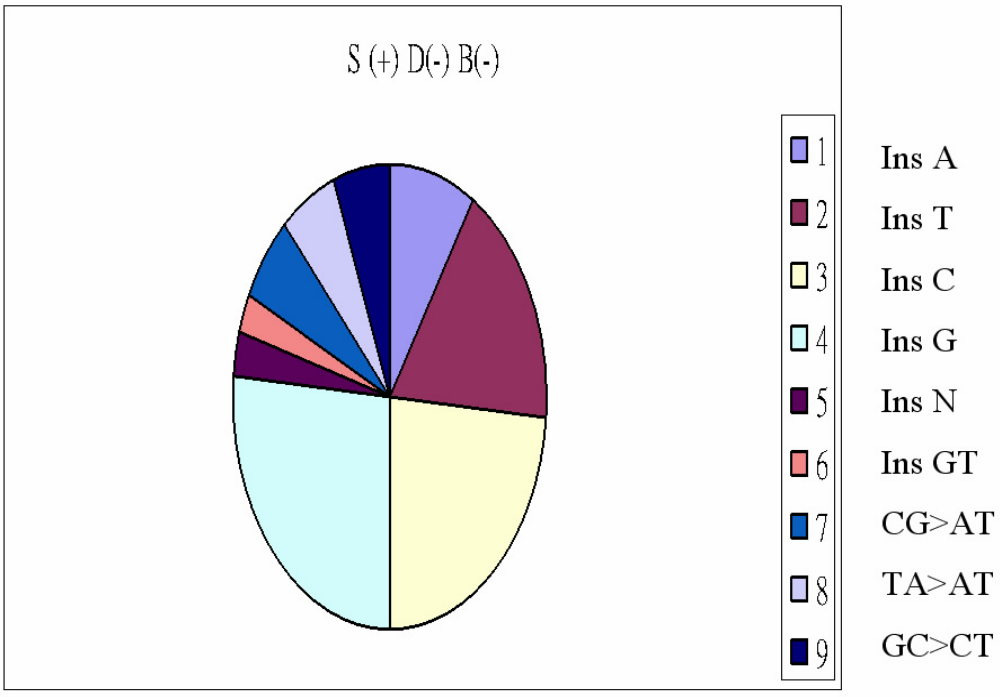
codon number

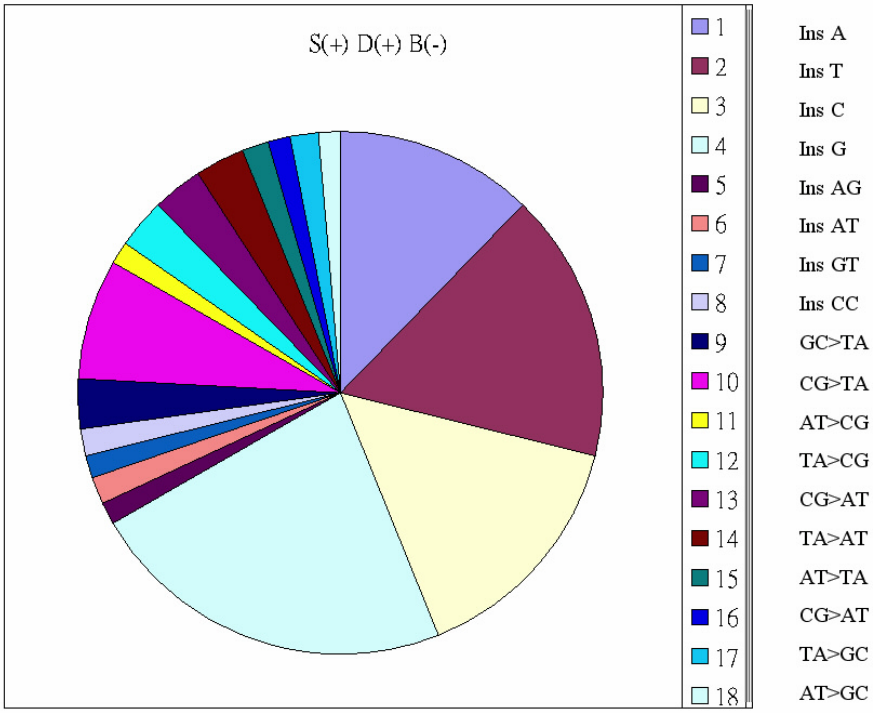


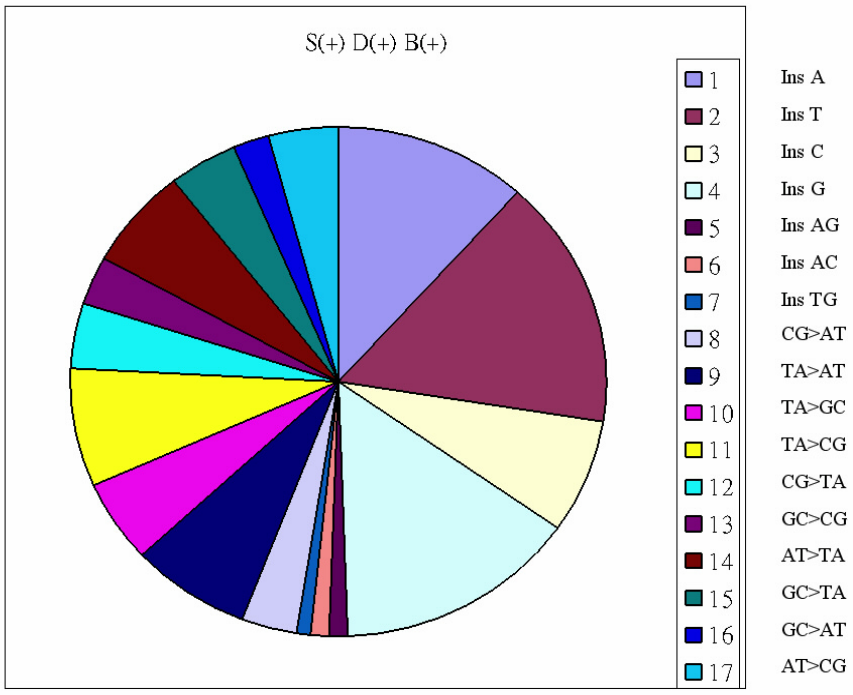
S: smoke; D: drinking; B: betal chewing; codon number: starting from the codon of exon 4 of p53.

Mutation pattern of p53 at codons 4, 6, 9


S(-) D(-) B(-)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Ins A
Ins T
Ins C
Ins G
Ins N
Ins AG
Ins CT
GC>CG
AT>TA
CG>TA
GC>TA
GC>AT
TA>AT
CG>AT
TA>GC
TA>CG


S (+) D(-) B(-)
1
2
3
4
5
6
7
8
9
Ins A
Ins T
Ins C
Ins G
Ins N
Ins GT
CG>AT
TA>AT
GC>CT


S(+) D(+) B(-)
1 Ins A
2 Ins T
3 Ins C
4 Ins G
5 Ins AG
6 Ins AT
7 Ins GT
8 Ins CC
9 GC>TA
10 CG>TA
11 AT>CG
12 TA>CG
13 CG>AT
14 TA>AT
15 AT>TA
16 CG>AT
17 TA>GC
18 AT>GC


S(+) D(+) B(+)
1 Ins A
2 Ins T
3 Ins C
4 Ins G
5 Ins AG
6 Ins AC
7 Ins TG
8 CG>AT
9 TA>AT
10 TA>GC
11 TA>CG
12 CG>TA
13 GC>CG
14 AT>TA
15 GC>TA
16 GC>AT
17 AT>CG

References

1.Cancer Registry Annual Report, Department of Health, Republic of China, 1996

2.YANG, C.S., Research on esophageal cancer in China: a review. *Cancer Res*., **40**, 2633-2644 (1980).

3. BLOT, W.J., and MCLAUGHLIN J.K. The changing epidemiology of esophageal cancer Semin. Oncol. 26 **(Suppl 15), 2-8 (1999)**

4 .YU, M.C., GARABRANT, D.H., PETERS, J.M. and MACK, T.M., Tobacco, alcohol, diet, occupation, and carcinoma of the esophagus. *Cancer Res*., **48**, 3843-3848 (1988).

5. GAO, Y.T., MCLAUGHLIN, J.K., BLOT, W.J., JI, B.T., BENICHOU, J., DAI, Q. and FRAUMENI, J.F., JR., Risk factors for esophageal cancer in Shanghai, China. I. Role of cigarette smoking and alcohol drinking. *Int. J. Cancer*, **58**, 192-196 (1994).

6.LEE JM , LEE YC , YANG SY, SHI WL, LEE CJ, LUH SP, CHEN CJ, HSIEH CY, WU MT , Genetic polymorphisms of p53 and GSTP1,but not NAT2,are associated with susceptibility to squamous-cell carcinoma of the esophagus , *Int. J. Cancer,* **89**, 458-464 (2000).

7. BROWN, L.M., HOOVER, R.N., GREENBERG, R.S., SCHOENBERG, J.B., SCHWARTZ, A.G., SWANSON, G.M., LIFF, J.M., SILVERMAN, D.T., HAYES, R.B., POTTERN, L.M. Are racial differences in squamous cell esophageal cancer explained by alcohol and tobacco use? J. Natl. Cancer Inst., **86**,1340-5 (1994)

8. CHENG, K.K., DUFFY, S.W., DAY, N.E., LAM, T.H. Oesophageal cancer in never-smokers and never-drinkers. Int. J. Cancer., ***60**, 820-2 (1995)*

9. GARIDOU, A., TZONOU, A., LIPWORTH, L., SIGNORELLO, L.B., KALAPOTHAKI, V., TRICHOPOULOS, D., Life-style factors and medical conditions in relation to esophageal cancer by histologic type in a low-risk population. Int. J. Cancer., **68**, 29529-9 (1996)

10. LAUNOY, G., MILAN, C.H., FAIVRE, J., PIENKOWSKI, P., MILAN, C.I., GIGNOUX, M., Alcohol, tobacco and oesophageal cancer: effects of the duration of consumption, mean intake and current and former consumption. Br. J. Cancer**.,** ***75**,1389-96 (1997)*

11. FRANCESCHI, S., TALAMINI, R., BARRA, S., BARON, A.E., NEGRI, E., BIDOLI, E. SERRAINO, D., LA VECCHIA, C., Smoking and drinking in relation to cancers of the oral cavity, pharynx, larynx, and esophagus in northern Italy. *Cancer* Res., **50**, 6502-7 (1990)

12. TAVANI, A., NEGRI, E., FRANCESCHI, S., LA VECCHIA, C., Risk factors for esophageal cancer in women in northern Italy. *Cancer. **72**,2531-6 (1993)*

13. KO, Y.C., HUANG, Y.L., LEE, C.H., CHEN, M.J., LIN, L.M. and TSAI, C.C., Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. *J. Oral Pathol. Med.*, **24**, 450-453 (1995).

14. SANKARANARAYANAN R., DUFFY, S.W., PADMAKUMARY, G., NAIR, S.M., DAY, N.E., and PADMANABHAN, T.K., Risk factors for cancer of the oesophagus in Kerala, India. *Int. J. Cancer*, **49**, 485-489, (1991).

15. WANG, C.K, HWANG, L.S., Phenol compounds of betel quid chewing juice. Food Sci, **20**, 458-471 (1993)

16. MILLER, J.A., MILLER, E.C., The metabolic activation and nucleic acid adducts of

naturally-occurring carcinogens: recent results with ethyl carbamate and the spice flavors safrole and estragole. *Brit. J. Cancer*., ***48**, 1-15 (1983)*

17. LIU, T.Y., CHEN, C.L., CHI, C.W., Oxidative damage to DNA induced by areca nut extract. Mut. Res., ***367**,25-31 (1996)*

18. NEWCOMB, P.A. and CARBONE, P.P., The health consequences of smoking. *Cancer. Med. Clin. North Am.*, **76**, 305-331 (1992).

19. VAN SCHOOTEN, F.J., GODSCHALK, R.W., BREEDIJK, A., MAAS, L.M., KRIEK, E., SAKAI, H., WIGBOUT, G., BAAS, P., VAN'T VEER, L. and VAN ZANDWIJK, N., 32P-postlabelling of aromatic DNA adducts in white blood cells and alveolar macrophages of smokers: saturation at high exposures. *Mut. Res*., **378**, 65-75 (1997).

20. YANG, Q., HERGENHAHN, M., WENINGER, A. and BARTSCH, H., Cigarette smoke induces direct DNA damage in the human B-lymphoid cell line Raji. *Carcinogenesis*, **20**, 1769-1775 (1999).

21. BROOKS, P.J., DNA damage, DNA repair, and alcohol toxicity--a review. Alcohol. *Clin. Exp. Res*., **21**,1073-82 (1997)

22. DAIKER, D.H., SHIPP, B.K., SCHOENFELD, H.A., KLIMPEL, G.R., WITZ, G., MOSLEN, M.T., and Ward, J.B., Jr., Effect of CYP2E1 induction by ethanol on the immunotoxicity and genotoxicity of extended low-level benzene exposure. *J. Toxicol. Environ. Health*, 59,181-96 (2000)

23. CASTELLI, E., HRELIA, P., MAFFEI, F., FIMOGANARI, C., FOSCHI, F.G., CAPUTO, F., CANTELLI-FORTI, G., STEFANINI, G.F. and GASBARRINI, G., Indicators of genetic damage in alcoholics: reversibility after alcohol abstinence. *Hepato-Gastroenterol*., **46**,1664-1668 (1999).

24. ANDERSON, L.M., SOULIOTIS, V.L., CHHABRA, S.K., MOSKAL, T.J., HARBAUGH, S.D. and KYRTOPOULOS, S.A., N-nitrosodimethylamine-derived O(6)-methylguanine in DNA of monkey gastrointestinal and urogenital organs and enhancement by ethanol. *Int. J. Cancer,* 66,130-134 (1996)

25. KNUUTILA S. AALTO Y. AUTIO K. BJORKQVIST AM. EL-RIFAI W. HEMMER S. HUHTA T. KETTUNEN E. KIURU-KUHLEFELT S. LARRAMENDY ML. LUSHNIKOVA T. MONNI O. PERE H. TAPPER J. TARKKANEN M. VARIS A. WASENIUS VM. WOLF M. ZHU Y. DNA copy number losses in human neoplasms ***American Journal** of **Pathology**. 155:683-94, 1999*

26. AHRENDT SA. CHOW JT. YANG SC. WU L. ZHANG MJ. JEN J. SIDRANSKY D. Alcohol consumption and cigarette smoking increase the frequency of p53 mutations in non-small cell lung cancer. *Cancer Research. 60:3155-9, 2000*

27. RALHAN R. SANDHYA A. MEERA M. BOHDAN W. NOOTAN SK. Induction of MDM2-P2 transcripts correlates with stabilized wild-type p53 in betel- and tobacco-related human oral cancer. *American Journal of Pathology*. 157:587-96, (2000)

28. Hussain SP, Harris CC. Molecular epidemiology of human cancer. *Rec Res Cancer Res* 1998;**154**: 22-36.

29. Hainaut P, Hollstein M. p53 and human cancer: the first 10,000 mutations. *Adv Cancer Res* 2000;**77**: 81-137.

30. Hollstein M, Sidransky D, Vogelstein B, et al. p53 mutations in human cancers. *Science*

1991;**253**: 49-53.
31. Greenblatt MS, Bennett WP, Hollstein M, et al. Mutations in the p53 tumor suppressor gene: clues to the cancer etiology and molecular pathogenesis. *Cancer Res* 1994;**54**: 4855-78
32. Soussi T, Legros Y, Lubin R, et al. Multifactorial analysis of p53 alteration in human cancer: a review. *Int J Cancer* 1994;**57**: 1-9.
33. Montesano R, Hollstein M, Hainaut P. Genetic alterations in esophageal cancer and their relevance to etiology and pathogenesis: a review. *Int J Cancer* 1996;**69**: 225-35.