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計畫名稱:p53 與 GSTP1 基因多型性與第十七、十八對染色體之遺傳因子喪失之關係

The Specific Allelic Deletion Profiles on Chromosomes 17 and 18 of Esophageal Squamous Cell Carcinomas Associated with Exposure to Different Environmental Agents

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一、中英文摘要:

關鍵詞:食道癌、 遺傳子喪失、腫瘤抑制基因

在癌症發展的過程中,腫瘤抑制基因的功能喪失,為一重要的機轉。每個不同的組織型別。為了尋找與食道癌發生有關的腫瘤抑制基因,我們運用雷射探取腫瘤切割技術,及位於 17、18 對染色體上 29 個微小星體標誌,我們發現食道癌的腫瘤在 17、18 對染色體上有多處遺傳子喪失的情形,其中有些地區與現在已知的腫瘤抑制基因的位置相吻合,而有些則未有明確的腫瘤抑制基因,如此顯示出在食道發生的過程中,有許多未知的腫瘤抑制基因參與其中。其中某些遺傳位置上的遺傳子喪失型態與環境毒物的暴露背景有關,在抽煙者中於 BRCA1(17q21)相對應的位置,有較高頻率產生遺傳子的喪失,在喝酒者則於 18p11.2 位置有較高頻率產生遺傳子喪失,而在有嚼檳榔者中,則在 p53 相對應的位置(17p12),有較高頻率產生遺傳子的喪失的現象,而 p53 基因多型性,則對這些遺傳子喪失的影響較不明顯。結論:食道癌在 17、18 對染色體有普遍產生遺傳子喪失的現象,而環境毒物的暴露可能與特殊的腫瘤抑制基因的喪失有關。

Abstract

Cigarette smoking, alcohol consumption, and areca chewing increase the risk of esophageal squamous cell carcinoma (EPC). To elucidate the molecular mechanisms induced by exposure to these agents, the present study investigated the relationship between genomic deletion profiles on chromosomes 17 and 18, on which certain tumor suppressor genes of EPC are located, and exposure to different environmental agents. The technique of laser capture microdissection was used to obtained pure tumor tissues, and allelotyping PCR, based on 29 microsatellite markers located on chromosomes 17 and 18, was used to define genomic deletion profiles. The results show that allelic loss at 17q21, the locus at which the *BRCA1* gene is located, was more frequently seen in tumors from cigarette smokers (56.7 % compared to 27.6 % in nonsmokers; $p=0.057$). Loss at 18p11.2 was significantly associated with frequent alcohol consumption (48 % compared to 17.7 % in non-frequent-consumers; $p< 0.05$). Reflecting the genetic status of p53, loss at 17p12 was more frequently found in

tumors in non-areca chewers (84.2 % compared to 41.7 % in areca chewers; $p<0.05$). These specific associations between allelic deletion profiles and environmental exposure suggest that different mechanisms are involved in carcinogenesis associated with different environmental carcinogens during EPC development.

Loss-of-function mutation in tumor suppressor genes (TSGs) is important in the development of hereditary and sporadic cancers. In the model proposed by Knudson, loss of TGS function usually results from two independent mutagenic "hits" on the TGS alleles(21). In EPC, LOH has been found in genomic areas encoding important TSGs, including *APC* and *MCC* (5q21), *p15* and *p16* (9p 21-23), *RB* (13q14), ING1 (13q33-34), *p53* (17p13), p73 (1p36.6) and *DCC* (18q21.1) (23-9). To explore possible differences in the tumorigenic mechanisms of EPC caused by exposure to different environmental agents, we correlated LOH profiles on chromosomes 17 and 18 in EPC with cigarette smoking, alcohol consumption, and areca chewing. Chromosomes 17 and 18 were chosen as the targets because many important TSGs for gastrointestinal tract cancer are located on these chromosomes. Furthermore, in this study, we purposely employed the technique of laser capture microdissection (LCM) (30), a powerful tool for obtaining pure tumor cells from infiltrating EPC, thus avoiding the problem of normal tissue contamination in LOH studies. Using these approaches, we have, for the first time, found specific allelic deletion in certain genomic areas associated with different environmental risk factors of EPC.

三・研究材料及方法

Study subjects.

In this study, 70 patients were randomly selected from this esophageal cancer cohort. All specimens were stored at -80°C until analysis. Institutional review board-approved informed consent was obtained from each patient prior to tissue acquisition.

Laser capture microdissection (LCM) and tumor DNA preparation.

LCM (30), performed to ensure the purity of the tumor sample, usually resulted in more than 90% purity of tumor cells. It was performed on immunostained slides using a PixCell laser capture microscope using the method suggested by the manufacturer (Arcturus Engineering, Mountain View, CA). Briefly, the stained, dehydrated tissue section was overlaid with a thermoplastic film mounted on an optically transparent cap, and the visually selected areas (tumor cells) bound to the membrane using short, low-energy laser pulses resulting in focal melting of the polymer. The cells was immersed in 50-100 ul, Tris buffer, pH 8.0, containing 0.5 mmol/L EDTA and 400 □g/ml proteinase K, and digested for 24 hours at 55°C. The enzyme was then heat-inactivated and the extract used directly for allelotyping PCR

(LOH detection).

Allelotyping PCR.

DNA from microdissected tumor specimens and normal samples (blood cell or normal esophageal tissue) was used for allelotyping PCR using fluorescent primers from the Applied Biosystems PRISM Linage Mapping Set-MD10 (PE Biosystems, Foster City, CA), comprising 29 polymorphic microsatellite markers on chromosomes 17 and 18 with an average spacing of 9.2 cM and average heterozygosity of 0.79. The marker loci were selected from the gene linkage map (33) on the basis of chromosomal location and heterozygosity. PCR amplification was carried out using 40 ng of genomic DNA, 0.4 U of Taq polymerase, 0.2 mM deoxynucleotides, and 2.5 mM $MgCl_2$ in a total reaction volume of 10 µl. The PCR conditions used were 95°C for 12 min to activate the Taq polymerase, followed by 50 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s, and a final elongation step at 72°C for 10 min. PCRs were run in a GeneAmp PCR 9600 thermocycler (PE Biosystems, Foster City, CA). For some markers yielding unsatisfactory amplification, the reaction conditions were modified and, if necessary, these particular markers were run singly. The PCR products were analyzed on a 377 ABI PRISM sequencer, and the fluorescent signals from the PCR products recorded and analyzed using GENOTYPER version 2.1 and GENESCAN version 3.1 software.

Definition of allelic loss (LOH).

For a given informative markers, we used our previous criteria (31-4) that the marker is considered to display LOH when a 3-fold or greater difference is seen in the relative allele intensity ratio between the tumor DNA and normal DNA.

Statistical analysis

To explore possible difference of genomic deletion profiles in EPC associated with different environmental exposures, the chi-squared test was used to evaluate associations between environmental exposure and the frequency of genetic deletions at individual genetic loci. The data were analyzed using the SAS statistical package.

四・結果與討論:

Table 1 shows the LOH frequencies for 24 markers on chromosomes 17 and 18. We deliberately excluded markers with less than 13 informative tumors because they provided inadequate statistic power for analysis. The markers representing the genomic loci of *p53* (17p12-13) displayed the highest LOH frequencies, ranging from 43.8% (*D17S1852*) to 69.6% (*D17S938*).

To increase the statistic power, markers locating at the same locus were considered together for analysis. Thus, individual tumors harboring at least one informative marker at the specific locus were considered to be "informative" and the

tumor was defined as "LOH" if it was found to show LOH in at least one marker at this locus. (Table 2) Cigarette smoking EPC patients showed a higher frequency of allelic deletion at 17q21 than non-cigarette smokers ($p= 0.057$). At the 18p11.2 locus (detected by the combined results for markers *D18S464* and *D18S53*), EPC patients who were frequent alcohol consumers had a higher frequency of LOH than non-alcohol drinkers ($p<0.05$). In contrast, LOH at locus 17p12 (combination of *D18S464* and *D18S53*) was less frequent in the tumors of EPC patients who chewed areca than in non-areca chewers ($p<0.05$).

Our study demonstrated that allelic deletion on chromosome 17 and 18 was very common in esophageal squamous cell carcinoma and that the genomic loci near *p53* (17p13) were the most vulnerable regions. These findings suggest the role of *p53* in our EPC patients, because mutation of the *p53* tumor suppressor gene is a hallmark of almost every cancer, including esophageal cancer (36-8), and, together with the LOH detected, comprise the two hits required to inactivate this gene during tumorigenesis. Other important TSGs on chromosomes 17 and 18, including the *BRCA1* (17q21) and *SMAD4* (18q21) genes and *DCC* (mapped to 18q 21.3) (40), are suspected of being involved in tumorigenesis, and our results support the role of these genes in EPC because of the high frequency of LOH detected.

Our findings support the idea that EPC's caused by exposure to different environmental agents may be linked to different molecular mechanisms. Different patterns of genomic deletion were associated with exposure to different environmental agents, as shown by preferential allelic deletion at 17q21 in cigarette smokers and at 18p11.2 in alcohol drinkers; while deletion at 17p12 was more frequently seen in non-areca chewers. One candidate TGS located at 17q21 is *BRCA1*, mutations of which account for a substantial portion of cases of familial breast cancer and ovarian cancer (42). One possible mechanism to explain preferential loss of the *BRCA1* locus in cigarette smokers developing EPC might come from the interaction between carcinogenic agents in cigarette and *BRCA1*. Exposure to polycyclic aromatic hydrocarbons (PAH), the most important carcinogens in cigarette smoke, can inhibit the expression of *BRCA1* (47); this inhibition is thought to occur through PAH-induced increased signaling via the aryl hydrocarbon receptor. Accordingly, PAH exposure resulting in reduced expression of *BRCA1* may serve as another hit of the Knudson's two hits, and, together with the *BRCA1* deletion (LOH), hampers the function of *BRCA1* leading to loss of DNA repair capacity, further contributing to a higher risk of esophageal cancer, as observed in our study.

We found that LOH at the *p53* locus was less frequent in areca chewers than in non-chewers. The tendency found in the present study is consistent with observations in oral cancer. *p53* mutation is detected in approximately 60% of oral cancer cases in

areas where cigarette smoke and alcohol consumption account for most of the attributable risks for oral cancer (51-2), but the frequency of *p53* mutation is relatively low in areca-related oral cancer. Observations in different areas where areca consumption is popular, including Sri Lanka, Papua New Guinea, India, and Taiwan have given similar results (5-20 % p53 mutation) (53-6).

Allelic deletion near 18p11.2 is commonly seen in cancers of the lung, prostate, breast, and esophagus, indicating that one, or more, putative TSGs are located in this region (41,57-8). Recently, *DAL-1*, a TSG located at 18p11.3 and differentially expressed in lung adenocarcinoma, has been identified (59). This gene is able to suppress tumor-outgrowth after introduction into a *DAL-1*-non-expressing lung cancer cell line (59). Our data demonstrated that alcohol-related esophageal cancer was associated with more frequent deletion in the locus near *DAL-1* (17p11.2), suggesting a relationship between *DAL-1* expression or mutation and exposure to ethanol. Table 1. Frequency of loss of heterozygosity (LOH) at loci on the chromosomes 17 and 18 in esophageal squamous cell carcinoma.

Table 1. Frequency of loss of heterozygosity (LOH) at loci on the chromosomes 17 and 18 in esophageal squamous cell carcinoma.

Marker	Locus	%LOH (No. LOH/No. Informative)
Chromosome 17		
D17S849	17p13.3	62.5 (10/16)
D17S831	17p13.3	57.6 (19/33)
D17S938	17p13.2	69.6 (16/23)
D17S1852	17p13.1	43.8 (7/16)
D17S799	17p12	47.8 (11/23)
D17S921	17p12	61.1 (11/18)
D17S1857	17p11.2	33.3 (5/15)
D17S1868	17q21.1	47.4 (9/19)
D17S787	17q21.31	37.1 (13/35)

D17S949	17q23.3	41.4 (12/29)
D17S785	17q25.1	16 (4/25)
D17S784	17q25.3	37.5 (6/16)
D17S928	17q25.3	40.5 (15/37)
Chromosome 18		
D18S59	18p11.32	29 (9/31)
D18S63	18p11.32	46.7 (14/30)
D18S 452	18p11.31	54.8 (23/42)
D18S464	18p11.22	38.5 (5/13)
D18S53	18p11.21	32.6 (14/43)
D18S 478	18q12.1	64.3 (9/14)
D18S1102	18q12.3	42.9 (15/35)
D18S474	18q21.1	55.6 (10/18)
D18S61	18q22.2	64.3 (9/14)
D18S 1161	18q23	38.1 (8/21)
D18S70	18q23	37.5 (9/24)

Table 2. Allelic loss at specific regions on the chromosomes 17 and 18 in relation to cigarette smoking, frequent alcohol drinking and areca chewing in esophageal squamous cell carcinoma.

Locus	Exposure status	%LOH (No. LOH/No. Informative)	P value
17q21*	Non-Smoker	26.7 (4/15)	0.057
	Smoker	56.7 (17/30)	
18p11.2**	Non-frequent drinker	17.7 (3/17)	0.044
	Frequent Drinker	48 (12/25)	
17p12***	Non-chewer	84.2 (16/19)	0.014
	Areca chewer	41.7 (5/12)	

*Combination of the results of the markers, *D17S1868*, *D17S787*, and *D17S944*.

**Combination of the results of the markers *D18S464* and *D18S53*.

***Combination of the results of the markers *D17S799* and *D17S921*.

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